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(54) Title: COMPOSITIONS AND METHODS FOR TREATING PAPILLOMAVIRUS-INFECTED CELLS (57) Abstract By virtue of the present invention, there is provided methods and compositions for interfering with the proliferation of cells infected and/or transformed by papillomaviruses. The processes and compositions of this invention may be used to treat any mammal, including humans. According to this invention, mammals are treated by the pharmaceutically acceptable administration of an E2 peptidomimetic to reduce the symptoms of the specific papillomavirus-associated disease, or to prevent their recurrence.		

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Compositions and Methods for Treating Papillomavirus-infected Cells

Background of the Invention

Papillomaviruses (PV) have been linked to widespread, serious human
5 diseases, especially carcinomas of the genital and oral mucosa. Tens of millions of
women suffer from human papilloma virus (HPV) infection of the genital tract.
Approximately 500,000 women worldwide develop cancer of the cervix each year and
it is the second most common cause of cancer deaths in women worldwide.
Approximately 90-95% of all cervical cancers may be linked to HPV.

10 In addition to causing severe cancerous conditions, Papillomaviruses more
commonly induce benign, dysplastic and malignant hyperproliferations of skin and
mucosal epithelium (see, for example, Mansur and Androphy, (1993) *Biochem
Biophys Acta* 1155:323-345; Shah and Howley (1996) *Fields Virology*, 3rd Ed., 2077-
2110; and Howley (1996) *Fields Virology*, 3rd Ed., 2045-2076, for reviews of the
15 molecular, cellular, and clinical aspects of the papillomaviruses). For example, a wide
variety of warts are found on human skin and are caused by the human papillomavirus
(HPV). Examples of such warts include common warts (*verruca vulgaris*), plantar
warts, palmar warts, planar warts (*verruca plana*), mosaic warts, and venereal warts
(*condyloma accuminatum*). These skin growths are unsightly, irritating, and
20 potentially carcinogenic, and their removal is desirable.

Over 70 different HPV types have been identified, and these different
papillomavirus types are known to cause distinct diseases, c.f., zur Hausen, (1996)
Biophysica Acta 1288:55-78; Pfister, (1987) *Adv. Cancer Res.*, 48:113-147; and
Syrjanen, (1984) *Obstet. Gynecol. Survey* 39:252-265. The HPVs that cause
25 anogenital warts can be further classified either high risk (such as HPV type 16 [HPV-
16] and HPV-18) or low risk (or HPV-6 and HPV-11) on the basis of the clinical
lesions with which they are associated and the relative propensity for these lesions to
progress to cancer. For example, HPV types 1 and 2 cause common warts, and types
6 and 11 cause warts of the external genitalia, anus and cervix. HPV's of the high risk
30 classification can be identified in the majority of cervical cancers, e.g., approximately

90% of human cervical cancers harbor the DNA of a high-risk HPV. Types 16, 18, 31 and 33 are particularly common; HPV-16 is present in about 50 percent of all cervical cancers.

The genetic organization of the papillomaviruses are defined by ten open
5 reading frames (ORFs) which are located on one strand of the viral DNA. These 10 genes are classified as early (E) and late (L) genes, depending on the time at which they are expressed in the viral life cycle, as shown in Figure 1. Several of the papillomavirus early genes code for regulatory proteins that are of particular interest. The high-risk HPV encoded E6 protein, for example, causes p53 degradation and the
10 E7 protein interacts with Rb and other pocket proteins (Howley et al., Fields Virology, Third Ed., Philadelphia: 1996). Notably, the E1 and E2 regulatory proteins play important roles in papillomavirus transcriptional regulation and viral DNA replication.

The biological life cycle of the papillomaviruses appears to differ from most
15 other viral pathogens. These viruses are believed to infect the basal or stem cells of the squamous epithelium. Rather than proceeding to a lytic infection in which viral replication kills the cell, viral DNA transcription and replication are maintained at very low levels until the infected epithelial cell migrates into the upper strata of the squamous epithelium. There, presumably in response to differentiation-specific
20 signals, the progression of viral transcription changes, viral DNA synthesis begins and infectious virions assemble. In this type of replication, hundreds of copies of the genomes are produced per cell, and these are packaged into progeny virions. High levels of E1 and E2 are observed in cells undergoing papillomavirus vegetative replication.

25 The papillomavirus E1 protein is the most conserved papillomavirus protein. The HPV-16 E1 protein is 649 amino acids long and has a molecular weight of 68-kd. The E1 protein is the only papillamovirus encoded DNA replication enzyme. All other replication enzymes, e.g., polymerases, primases, etc. are provided by the infected host cell. The E1 protein is a nuclear phosphoprotein and ATP-dependent
30 DNA helicase that binds to the origin of DNA replication (ori), thus initiating viral plasmid replication (Howley 1996). The minimal DNA binding domain of the E1

proteins is found in the amino terminus. The HPV-16 E1 carboxy-terminus contains a domain that is necessary and sufficient for interaction with the E2 protein. This domain also includes an ATP binding pocket similar to that found in SV40 large T antigen (LgT).

5 Interestingly, the papillomavirus E1 protein shares many structural and functional similarities with the large T antigen (LgT) of simian virus (SV40), the replication protein of that virus. Both E1 and LgT bind a region of dyad symmetry within the DNA replication origin and have ATP-dependent DNA helicase activity that can catalyze the unwinding of ori-containing double-stranded DNA templates
10 (Smelkova and Borowiec, *Journal of Virology*, 1997, 71, 8766; Chen et al., *Journal of Virology*, 1997, 72, 2567). One important difference between the two proteins is that LgT is the only viral protein required for initiation of SV40 replication (Bullock et al., *Critical Reviews in Biochemistry and Molecular Biology*, 1997, 32, 503), whereas E1 is efficiently recruited to the viral DNA replication origin, only through interaction
15 with the virus encoded E2 protein (see below).

The papillomavirus E2 protein is a critical regulator of both viral DNA replication and gene expression. HPV-16 E2 is 365 amino acids long and has a molecular weight of 38-kD. E2 proteins are composed of two well-conserved functional domains, as shown in Figure 2. The E2 carboxy-terminus includes a DNA
20 binding domain that binds as a dimer to the ACCN6GGT recognition sequence (Andropy et al., *Nature*, 1987, 325, 70). The E2 amino-terminus features a transcriptional activation domain that regulates viral gene expression and interacts with components of the host cell apparatus. The E2 N-terminus also interacts with the E1 protein. These amino-terminal and the carboxy-terminal domains are connected
25 by a hinge region that is dispensable for both replication and transcriptional activation.

Genetic studies have revealed that stable BPV-1 plasmid replication requires the expression of the viral E1 and E2 genes (DelVecchio et al., *Journal of Virology*, 1992, 66, 5949; Di Maio et al., *EMBO*, 1988, 7, 1197; Sarver et al., *Journal of*
30 *Virology*, 1984, 52, 377). Though E1 and E2 are both essential factors for stable DNA replication, it appears that only E1 is directly involved in plasmid replication

(DiMaio and Settlement 1986, Ustav et al. 1991). However, extensive research over the past ten years has been conducted to elucidate the role of E2 in DNA replication, and the results of these numerous studies suggest that (1) HPV-16 E2 transactivation functions are independent of and separable from its E1 interaction (i.e., replication activity), (2) E1 binding is necessary for E2 stimulation of DNA replication. E2, by binding to ACC 6GT recognition sequences that flank the viral DNA replication origin, recruits E1 to the origin. In this way, E2 effects initiation of the formation of the viral replication complex.

Thus, since the formation of an E1-E2 complex is necessary for stimulation of DNA replication, moieties that can block the formation of this complex could serve to inhibit viral DNA replication and thus the proliferation of harmful conditions as described above.

Towards this end, European patent application 302,758 refers to the use of modified forms of E2 protein that bind to, and block, E2 binding sites on papillomavirus DNA without resulting in trans-activation. That application also refers to repression of E2 activation through the use of DNA fragments that mimic E2 binding sites, and thus bind with E2 trans-activators, making them unavailable for binding to E2 sites on the viral DNA.

Additionally, U.S. Patent 5,219,990 describes the use of E2 trans-activation repressors which interfere with normal functioning of the native full-length E2 transcriptional activation protein of the papillomavirus. However, the E2 trans-activation repressors of the '990 patent are proteins that dimerize with the full-length native E2 protein to form inactive heterodimers, thus interfering with the formation of active homodimers comprising full-length native E2 polypeptides and thereby repressing papillomavirus transcription and replication. The E2 trans-activation repressors are described as fragments of the E2 polypeptide in which the dimerization function has been separated from its DNA binding function, e.g., the E2 trans-activation repressors includes at least the dimerization region, but less than the DNA binding domain, of the E2 polypeptide.

Although these references are directed towards the blocking of complex formation, each of these references describes the use of *large biological molecules*,

namely proteins, to achieve this goal. Clearly, there remains a need for the development of *simpler, preferably cell-permeable, small molecule therapeutics* capable of treating a papillomavirus-induced condition, lessening the severity of such condition, or preventing the occurrence of such condition.

Summary of the Invention

One aspect of the present invention provides a method of treating, e.g., lessening the severity or preventing the occurrence of a papillomavirus-induced condition, such as a papillomavirus-induced lesion. In general, the subject method comprises administering to an animal, e.g. a human, infected with a papillomavirus a pharmaceutical preparation comprising a therapeutically effective amount of a small organic molecule which can inhibit E1-E2 interaction. In preferred embodiments, the inhibitor has a molecular weight of less than 10,000 amu, more preferably less than 7500 amu, 5000 amu, and even more preferably less than 3000 amu. For instance, the E2 inhibitor can be either (i) an E2 peptide or peptidomimetic, preferably corresponding in length to a 3-25 mer, e.g., in certain embodiments, containing a core sequence of R-X(4)-E-X(5)-X(6)-X(7) and in other embodiments, including the core sequence W-X(1)-X(2)-X(3)-R-X(4)-E-X(5)-X(6)-X(7)-X(8)-X(9)-X(10)-A-X(11), or (ii) a gene construct for expressing the E2 peptide. The E2 peptide, peptidomimetic or gene construct is formulated in the pharmaceutical preparation for delivery into PV-infected cells of the animal.

In preferred embodiments, the subject method is used to treat a human who is infected with a human papillomavirus (HPV), particularly a high risk HPV such as HPV-16, HPV-18, HPV-31 and HPV-33. In other preferred embodiments, treatment of low risk HPV conditions, e.g., particular topical treatment of cutaneous or mucosal low risk HPV lesions, is also contemplated.

The subject method can be used to inhibit pathological progression of papillomavirus infection, such as preventing or reversing the formation of warts, e.g. Plantar warts (verruca plantaris), common warts (verruca plana), Butcher's common warts, flat warts, genital warts (condyloma acuminatum), or epidermodysplasia verruciformis; as well as treating papillomavirus-infected cells which have become, or are at risk of becoming, transformed and/or immortalized, e.g. cancerous, e.g. a laryngeal papilloma, a focal epithelial, a cervical carcinoma, or as an adjunct to chemotherapy, radiation, surgical or other therapies for eliminating residual infected or pre-cancerous cells.

Yet another aspect of the invention relates to a pharmaceutical preparation comprising a therapeutically effective amount of an E2 peptidomimetic, formulated in the pharmaceutical preparation for delivery into PV-infected cells of an animal. In preferred embodiments, the polypeptide is formulated as a liposome.

5 As will be appreciated by one of ordinary skill in the art, the compositions and preparations described herein can also be utilized serially or in combination with conventional therapeutic agents or regimens including, but not limited to, salicylic acid, podophyllotoxin, retinoic acid, surgery, laser therapy, radiation, and cryotherapy.

The practice of the present invention will employ, unless otherwise indicated,
10 conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*,
15 Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical*
20 *Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of*
25 *Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Brief Description of the Figures

Figure 1. Genomic Map of HPV-16. The genome is a double-stranded circular DNA molecule containing 7.904 base pairs. Transcription occurs in a clockwise manner. Only one promoter has been identified thus far. Open reading frames deduced from the DNA sequences are designated E1-E7 and L1-L2. The viral long control region (LCR) contains transcriptional and replication regulatory elements. Diagram courtesy of Peter Howley (Fields Virology, Third Edition 1996).

Figure 2. Functional domains of the papillomavirus E2 protein. The E2 regulatory protein is composed of two well-conserved domains. Transcriptional activation and E1 interaction functions have been localized to a domain spanning aa1-190. This domain encompasses the E39 residues which is critical for viral DNA replication. The E2 DNA binding and dimerization domain spans approximately the carboxy-terminal 100 amino acids. The amino-terminal and carboxy-terminal domains are connected by a hinge region.

Figure 3, Panels A, B and C. E2 stimulates replication of HPV16 *in vitro*.

A. Replication of the HPV16 origin containing plasmid p16ori, a Bluescript KS+ plasmid containing HPV16 genomic sequences (7838-139), using 293 cell extract and increasing amounts of EE-16E1 (10-50 ng) in the presence (lanes 8-13) or absence (lanes 2-7) of HPV16 E2. The reaction in lane 1 contained no HPV16 E1 or E2. Replicative intermediates (RI), Form I and Form II DNA analogous to those observed by Kuo et al. (18) are indicated. B. *In vitro* HPV16 DNA replication is origin dependent. Minimal replication of the Bluescript KS+ plasmid that does not contain HPV16 origin sequences is observed under conditions identical to those described in A. C. Phosphorimager quantitation of the replication experiments shown in Figures 1A and B.

Figure 4, Panels A and B. Interaction of the E2 amino terminus with E1 is required for HPV16 DNA replication *in vitro*. A. Replication of p16ori (40ng) was determined in the presence of 293 cell extract, 20ng EE-E1, and increasing amounts of wild type (lanes 2-9) or E1 interaction defective E39A HPV16 E2 (lanes 10-16). B. Phosphorimager quantitation of the replication experiments shown in Figure 4A.

Figure 5A. Derivation of candidate HPV16 E1-E2 interaction inhibitory peptides based on conserved residues within the E2 amino terminus. Mutagenesis

studies identified residue E39 of HPV16 E2 as critical for interaction with E1. The upper panel shows an alignment of conserved proximal sequences in other papillomavirus E2 proteins. A deduced consensus sequence is shown in the middle panel. The lower panel shows the sequences of the synthetic peptides derived from this portion of HPV16 E2. WP peptides retain the conserved glutamic acid at the position analogous to E39 of HPV16 E2, whereas the MP peptides contain a substituted alanine at this position.

Figure 5B. Sequence alignment of various papillomavirus E2 proteins.

Figure 6, Panels A and B. Peptides derived from the HPV16 E2 amino terminus inhibit *in vitro* interaction between HPV16 E1 and E2. A. Indicated amounts of epitope tagged HPV16 E1 (EE-E1) and HPV16 E2 were incubated in NET-gel buffer in the presence of increasing concentrations of the 23-mer (E2N-WP23; upper panel lanes 2-9) of the 15-mer (E2N-WP15; lower panel lanes 2-9). Binding of EE-E1 to E2 was determined by immunoprecipitation using an anti-EE epitope monoclonal antibody, and E2 was detected using anti-HPV16 E2C antiserum (31). Precipitation of E1 in each reaction was confirmed by probing the same blot with polyclonal antisera recognizing HPV16 E1. Increasing amounts of E2N-MP23 and E2N-MP15 peptides were used in lanes 10-13 (upper and lower panels). Lane 14 represents the level of interaction observed between the HPV16 E39A mutant protein and EE-E1. Input wild type and E39A mutant HPV16 E2 proteins are shown in lanes 15 and 16. B. Phosphorimager quantitation of the E2 reactive bands from the experiments shown in panel A.

Figure 7, Panels A, B and C. Peptides derived from the HPV16 E2 amino terminus inhibit replication of HPV16 *in vitro*. A and B. *In vitro* replication of HPV16 DNA replication was determined using 40ng EE-E1 and 6ng HPV16 E2 in the presence of increasing amounts of E2N-WP23 or E2N-WP15 (Figures 7A and 7B, respectively). Negative controls included reactions with only 293 cell extract and p16ori (lane 1, 7A and 7B), EE-E1 in the absence of E2 (lane 2, A and B), or complete replication reactions containing increasing concentrations of E2N-WP23 and E2N-WP15 (Figures 7 A and B, lanes 11-14). C. Phosphorimager quantitation of the replication experiments shown in Figures 7 A and B. The amount of replication

relative to that in lanes 3, in the absence of inhibitory peptide, is plotted logarithmically as a function of peptide concentration.

Figure 8, Panels A, B and C. E2N-WP15 inhibits E1-E2 interaction and *in vitro* DNA replication of HPV11. A. Inhibition of E1-E2 interaction was compared between HPV16 E1 and HPV16 E2 (lanes 1-8), HPV11 E1 and HPV16 E2 (lanes 9-18), and between HPV11 E1 and HPV11 E2 (lanes 19-26). Epitope tagged E1 proteins were precipitated using anti-EE epitope monoclonal antibody, and associated E2 proteins were detected by Western blot using anti- E2 antiserum. Negative control reactions containing EE-E1 with no E2 are shown in lanes 1, 9, and 19. The amounts of input HPV16 E2 and E39A used in lanes 1-16 are shown in lanes 17 and 18. The input amount of E2 used in the HPV11 E2 experiment (lanes 19-25) is shown in lane 26. B. *In vitro* replication of the HPV11 origin plasmid p11ori(7874-99) using increasing amounts of purified HPV11 EE-E1 in the presence (lanes 6-9) or absence (lanes 2-5) of 6ng HPV16 E2. The reaction in lane 1 contained no HPV proteins. C. The ability of E2N-WP15 to inhibit *in vitro* replication of the HPV11 or using HPV11 EE-E1 and HPV16 E2 was tested (lanes 4-8). Effects of equivalent concentrations of E2N-MP15 peptide were also determined (lanes 9-11).

Figure 9. Kinetics of interaction between HPV16 EE-E1 and E2 in the binding assay *in vitro*. *In vitro* E1-E2 binding assays were carried out for the indicated times (lanes 2-7), and E1-E2 complexes were determined by immunoprecipitation using anti-EE monoclonal antibody, followed by Western blot using antiserum to the C- terminus of HPV16 E2. In parallel reactions, E2N-WP15 peptide (50μM final concentration) was added to the E1-E2 binding reaction at the indicated times (lanes 8-12), and reactions were continued through termination at 60 minutes.

Figure 10. Amino acid sequences of HPV-16 E2 derived 15-amino acid peptides containing single amino acid alanine substitutions. Wild-type peptide is denoted as "WP15". For mutant peptides, name describes position and identity of original residue for which an alanine was substituted. Each substituted residue is underlined.

Figure 11. In vitro inhibition of HPV-16 E1-E2 interaction by alanine-scanning mutant E2 peptides. Binding assays were performed in the presence of 0, 10, 20 or 40 μ M peptide. Bound E2 was detected by Western Analysis. (A) Autoradiography data. (B) Intensities of bands were determined by NIH Image Program. For each peptide, the positive control (binding assay in the absence of peptide) was standardized to 100% E2 binding.

Figure 12. Alignment of conserved E39-proximal amino acid sequences of papillomavirus E2 proteins. Conserved residues are highlighted by the boxes.

Figure 13. Amino acid sequence of HPV-16 E2 fusion peptides. (A) A fusion peptide containing thirteen amino acids derived from the basic translocating domain of the Tat protein (aa48-60 Y, followed by WF115 was synthesized. These moieties were separated by a Gly-Gly linker. This peptide is referred to as HTat-WP". (B) Another fusion peptide containing 17 amino acids derived from the third homeodomain of Antennapedia(aa42-58) protein, followed by the WP15 (Ant-WP) was tested for inhibition of E1-E2 interaction.

Figure 14. *In vitro* inhibition of HPV-16 E1-E2 interaction by Tat- and Ant-WP fusion peptides. Assays were performed in the presence of 0, 10, 20, or 40 μ M peptide. Western Analysis was performed and bands were detected by E2-antibody. (A) Autoradiography data. (B) Intensities of bands were determined by NIH Image Program. For each peptide, the positive control (E1-E2 binding in the absence of peptide) was normalized to 100% E2 binding.

Detailed Description of the Invention

I. Overview

According to the present invention, there is provided methods and compositions for interfering with papillomavirus DNA replication. The processes and compositions of this invention may be used to treat any mammal, including humans. According to this invention, mammals are treated by the pharmaceutically acceptable administration of an E2-derived peptide or peptidomimetic (herein collectively "E2 peptidomimetic) or other small molecule inhibitor of E2 function, either directly or by

gene transfer techniques, to reduce the symptoms of the specific papillomavirus-associated disease, or to prevent their recurrence.

As discussed above, the papillomaviruses (PV) are infectious agents that can cause benign epithelial tumors, or warts, in their natural hosts. Infection with specific human papillomaviruses (HPV) is also associated with human epithelial malignancies, including malignancies of the uterine cervix, genitalia, skin and, though less frequently, other sites.

As described in the appended examples, and as shown in detail in Figures 6, 7, and 8, we have discovered that mutation of the conserved glutamic acid residue at position 39 of HPV16 E2 to alanine (E39A) disrupts its E1 interaction activity and its replication function in transient replication assays, but does not affect E2 transcriptional activation (Sakai et al. (1996) *J Virol* 70:1602-1611). This E39A mutation also disrupts replication activity of HPV16 E2 in HPV16 *in vitro* DNA replication. On this basis, we designed peptides derived from HPV16 E2 sequences flanking the E39 residue and tested the ability of these peptides to inhibit interaction between HPV16 E1 and E2 *in vitro*. The inhibitory activity of these peptides was specific, since analogous peptides that substituted alanine for the E39 residue did not inhibit interaction. The peptide E2N-WP15 (described below), for example, effectively inhibited HPV16 E1-E2 interaction. This peptide also inhibited *in vitro* replication of HPV16 DNA. The efficacy of E2N-WP15 was not exclusive to HPV16: this peptide also inhibited interaction of HPV11 E1 with the E2 proteins of both HPV11 and HPV16, and inhibited *in vitro* replication with these same combinations of E1 and E2 proteins. These results constitute the first demonstration that inhibition of E1-E2 interaction is sufficient to prevent HPV DNA replication *in vitro*.

In order to further define the E1-E2 interaction and design more efficient peptides peptidomimetics, an alanine scanning approach was used to determine specific residues within the E2N-WP15 peptide that are important for the inhibition of E1-E2 interaction. In general, for HPV-16 E2, residues R37, L38, E39, and I42 were found to be important for the inhibitory activity of the peptide for HPV-16. Figure 12 depicts the alignment of conserved E39-proximal amino acid sequences, in which conserved residues are highlighted by the boxes. This further demonstrates the

importance of R37 and E39 in the E1-E2 interaction, and together with the information obtained from HPV-16 E2, enables the development of core peptides and peptidomimetics to be used in the inhibition of the E1-E2 interaction, as described herein.

5 In preferred embodiments, the present invention provides a peptide, or peptidomimetic that inhibits the biological activity of the PV E2 protein, such as E2 dependent replications. The peptide/peptidomimetic can, in certain, preferred embodiments range in size from 3-25 amino acid residues. In certain embodiments, the E2 inhibitor of the present invention includes a core structure having the formula:
10 R-X(4)-E-X(5)-X(6)-X(7) or a mimetic thereof. In certain other embodiments, the peptide may include a core structure having the formula: W-X(1)-X(2)-X(3)-R-X(4)-E-X(5)-X(6)-X(7)-X(8)-X(9)-X(10)-A-X(11), wherein:

W represents a tryptophan residue, or an analog thereof;

R represents an arginine residue, or an analog thereof;

15 E represents a glutamic acid residue, or an analog thereof;

A represents an alanine residue, or an analog thereof;

X(1) represents an amino acid residue having a polar sidechain, such as arg, asn, asp, cys, glu, gln, his, lys, ser, thr or tyr, or an analog thereof;

20 X(2), X(5), X(6), X(7) and X(8) each, independently, represent an amino acid residue having a neutral sidechain, such as ala, asn, cys, gln, gly, his, ile, leu, met, phe, pro, ser, thr, trp, tyr, or val, or an analog thereof;

X(3) represents an amino acid residue having a hydrophobic sidechain, such as ala, gly, ile, leu, met, phe, pro, trp, tyr, or an analog thereof;

25 X(4) and X(10) each, independently, represent an amino acid residue having a basic sidechain, such as arg, his or lys, or a neutral sidechain, such as ala, asn, cys, gln, gly, his, ile, leu, met, phe, pro, ser, thr, trp, tyr or val, or an analog thereof;

X(9) represents an amino acid residue having an aromatic sidechain, such as his, phe, trp or tyr, or an analog thereof; and

X(11) represents an amino acid residue having a basic sidechain, such as arg, his or lys, or an analog thereof.

It will also be appreciated that other core structures having a greater number of residues than the abovedescribed six membered core, and fewer residues than the abovedescribed fifteen membered core, is also contemplated by the present invention.

In certain embodiments of the subject E2 peptidomimetic: X(1), X(2), X(3), X(9), X(10) and X(11) are as defined above: X(4) represents an amino acid residue having a basic sidechain or a neutral hydrophobic sidechain, such as ala, gly, ile, leu, met, phe, pro, trp, tyr or val, or an analog thereof; X(5) represents an amino acid residue having a neutral polar sidechain, such as asn, cys, gln, his, ser or thr, or an analog thereof; X(6) and X(7) each independently represent an amino acid residue having neutral aliphatic sidechain, such as ala, gly, ile, leu, or val, or an analog thereof; and X(8) represents an amino acid residue having a large hydrophobic sidechain, such ile, leu, met, phe, trp, or tyr or an analog thereof.

In preferred embodiments of the subject E2 peptidomimetic: X(1) represents gln, lys, tyr, cys or thr or an analog thereof, and more preferably gln, lys or cys or an analog thereof; X(2) represents leu, thr, cys, tyr, his, ala or cys or an analog thereof; X(3) represents met, leu, ile, val, thr or arg or an analog thereof, or an analog thereof, and more preferably met, leu, ile or val or an analog thereof; X(4) represents leu, lys, his, tyr, trp, arg, val, ile, met, gln or thr or an analog thereof, and even more preferably leu, lys, his, tyr, trp, arg, val, ile or met or an analog thereof; X(5) represents gln, ala, ser, cys, asn or tyr or an analog thereof; X(6) represents ala, val or thr or an analog thereof, and even more preferably ala or val or an analog thereof; X(7) represents leu, ile or val or an analog thereof, and even more preferably leu or ile or an analog thereof; X(8) represents leu, tyr, phe, met or gln or an analog thereof; X(9) represents tyr, his or phe or an analog thereof, and even more preferably tyr or his or an analog thereof; X(10) represents lys, tyr, thr or ala or an analog thereof, and even more preferably lys or tyr or an analog thereof; and X(11) represents arg or lys or an analog thereof.

In other preferred embodiments, the peptide or peptidomimetic represents the six sequence core wherein X(4) represents leu, trp, gln, his, met, or thr, or an analog

thereof; X(5) represents cys. asn. gln or ser, or an analog thereof; X(6) represents ala, val. or thr, or an analog thereof; and X(7) represents ile. leu. val. or met. or an analog thereof.

Moreover, Applicants believe that the subject peptides are the first example of
5 any small molecule capable of inhibiting E1-E2 interaction. Prior to Applicants' discovery, there was no evidence to suggest that a small molecule would be capable of inhibiting that complex and thereby be useful for treatment of PV-related diseases. In fact, prior studies have actually suggested that multiple large domains were required for the necessary interaction (see, for example, Abroi, A., R. Kurg, and M. Ustav,
10 1996, J. Virol. 70: 6169-6179; Breiding, D.E., M.J. Grossel, and E.J. Androphy, 1996, Virology 221: 34-43; Grossel, M.J., F. Sverdrup, D.E. Breiding, and E.J. Androphy, 1996, J. Virol. 70: 7264-7269; Sakai, H., T. Yasugi, J.D. Benson, J.J. Dowhanick, and P.M. Howley, 1996, J. Virol. 70: 1602-1611; Winokur, P.L. and A.A. McBride, 1992, EMBO J. 11: 4111-4118). Thus, the present invention provides a general approach to
15 the treatment of PV-related diseases utilizing peptidyl and non-peptidyl small molecule inhibitors of E1-E2 interaction.

II. Definitions

For convenience, certain terms employed in the specification, examples, and
20 appended claims are collected here.

The term "heteroatom" as used herein means an atom of any element other than carbon or hydrogen. Preferred heteroatoms are boron, nitrogen, oxygen, phosphorus, sulfur and selenium.

The term "alkyl" refers to the radical of saturated aliphatic groups, including
25 straight-chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups. In preferred embodiments, a straight chain or branched chain alkyl has 30 or fewer carbon atoms in its backbone (e.g., C₁-C₃₀ for straight chain, C₃-C₃₀ for branched chain), and more preferably 20 or fewer. Likewise, preferred cycloalkyls have from

3-10 carbon atoms in their ring structure, and more preferably have 5, 6 or 7 carbons in the ring structure.

Moreover, the term "alkyl" (or "lower alkyl") as used throughout the specification, examples, and claims is intended to include both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, a halogen, a hydroxyl, a carbonyl (such as a carboxyl, an alkoxy carbonyl, a formyl, or an acyl), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an alkoxy, a phosphoryl, a phosphonate, a phosphinate, an amino, an amido, an amidine, an imine, a cyano, a nitro, an azido, a sulfhydryl, an alkylthio, a sulfate, a sulfonate, a sulfamoyl, a sulfonamido, a sulfonyl, a heterocyclyl, an aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate. For instance, the substituents of a substituted alkyl may include substituted and unsubstituted forms of amino, azido, imino, amido, phosphoryl (including phosphonate and phosphinate), sulfonyl (including sulfate, sulfonamido, sulfamoyl and sulfonate), and silyl groups, as well as ethers, alkylthios, carbonyls (including ketones, aldehydes, carboxylates, and esters), -CF₃, -CN and the like. Exemplary substituted alkyls are described below. Cycloalkyls can be further substituted with alkyls, alkenyls, alkoxy, alkylthios, aminoalkyls, carbonyl-substituted alkyls, -CF₃, -CN, and the like.

The term "aralkyl", as used herein, refers to an alkyl group substituted with an aryl group (e.g., an aromatic or heteroaromatic group).

The terms "alkenyl" and "alkynyl" refer to unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively.

Unless the number of carbons is otherwise specified, "lower alkyl" as used herein means an alkyl group, as defined above, but having from one to ten carbons, more preferably from one to six carbon atoms in its backbone structure. Likewise, "lower alkenyl" and "lower alkynyl" have similar chain lengths. Preferred alkyl

groups are lower alkyls. In preferred embodiments, a substituent designated herein as alkyl is a lower alkyl.

The term "aryl" as used herein includes 5-, 6- and 7-membered single-ring aromatic groups that may include from zero to four heteroatoms, for example, 5 benzene, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyridine, pyrazine, pyridazine and pyrimidine, and the like. Those aryl groups having heteroatoms in the ring structure may also be referred to as "aryl heterocycles" or "heteroaromatics." The aromatic ring can be substituted at one or more ring positions with such substituents as described above, for example, halogen, azide, alkyl, aralkyl, 10 alkenyl, alkynyl, cycloalkyl, hydroxyl, alkoxy, amino, nitro, sulfhydryl, imino, amido, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, sulfonamido, ketone, aldehyde, ester, heterocyclyl, aromatic or heteroaromatic moieties, $-CF_3$, $-CN$, or the like. The term "aryl" also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common 15 to two adjoining rings (the rings are "fused rings") wherein at least one of the rings is aromatic, e.g., the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocyclyls.

The terms "heterocyclyl" or "heterocyclic group" refer to 3- to 10-membered ring structures, more preferably 3- to 7-membered rings, whose ring structures include 20 one to four heteroatoms. Heterocycles can also be polycycles. Heterocyclyl groups include, for example, thiophene, thianthrene, furan, pyran, isobenzofuran, chromene, xanthene, phenoxathiin, pyrrole, imidazole, pyrazole, isothiazole, isoxazole, pyridine, pyrazine, pyrimidine, pyridazine, indolizine, isoindole, indole, indazole, purine, quinolizine, isoquinoline, quinoline, phthalazine, naphthyridine, quinoxaline, 25 quinazoline, cinnoline, pteridine, carbazole, carboline, phenanthridine, acridine, pyrimidine, phenanthroline, phenazine, phenarsazine, phenothiazine, furazan, phenoxazine, pyrrolidine, oxolane, thiolane, oxazole, piperidine, piperazine, morpholine, lactones, lactams such as azetidinones and pyrrolidinones, sultams, sultones, and the like. The heterocyclic ring can be substituted at one or more 30 positions with such substituents as described above, as for example, halogen, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino,

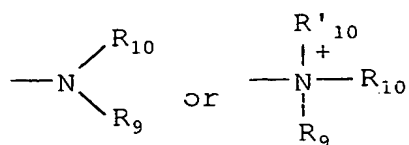
amido, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, -CF₃, -CN, or the like.

The terms "polycyclyl" or "polycyclic group" refer to two or more rings (e.g., cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocyclyls) in which two or more carbons are common to two adjoining rings, e.g., the rings are "fused rings". Rings that are joined through non-adjacent atoms are termed "bridged" rings. Each of the rings of the polycycle can be substituted with such substituents as described above, as for example, halogen, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, -CF₃, -CN, or the like.

The term "carbocycle", as used herein, refers to an aromatic or non-aromatic ring in which each atom of the ring is carbon.

As used herein, the term "nitro" means -NO₂; the term "halogen" designates -F, -Cl, -Br or -I; the term "sulfhydryl" means -SH; the term "hydroxyl" means -OH; and the term "sulfonyl" means -SO₂-.

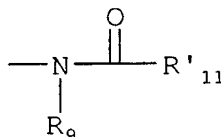
The terms "amine" and "amino" are art-recognized and refer to both unsubstituted and substituted amines, e.g., a moiety that can be represented by the general formula:



wherein R₉, R₁₀ and R'₁₀ each independently represent a hydrogen, an alkyl, an alkenyl, -(CH₂)_m-R₈, or R₉ and R₁₀ taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure; R₈ represents an aryl, a cycloalkyl, a cycloalkenyl, a heterocycle or a polycycle; and m is zero or an integer in the range of 1 to 8. In preferred embodiments, only one of R₉ or R₁₀ can be a carbonyl, e.g., R₉, R₁₀ and the nitrogen together do not form an imide. In even more preferred embodiments, R₉ and R₁₀ (and optionally R'₁₀) each

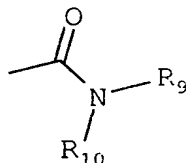
independently represent a hydrogen, an alkyl, an alkenyl, or $-(CH_2)_m-R_8$. Thus, the term "alkylamine" as used herein means an amine group, as defined above, having a substituted or unsubstituted alkyl attached thereto, i.e., at least one of R_9 and R_{10} is an alkyl group.

- 5 The term "acylamino" is art-recognized and refers to a moiety that can be represented by the general formula:



wherein R_9 is as defined above, and R'_{11} represents a hydrogen, an alkyl, an alkenyl or $-(CH_2)_m-R_8$, where m and R_8 are as defined above.

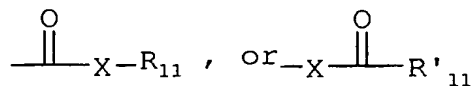
- 10 The term "amido" is art recognized as an amino-substituted carbonyl and includes a moiety that can be represented by the general formula:



wherein R_9 , R_{10} are as defined above. Preferred embodiments of the amide will not include imides which may be unstable.

- 15 The term "alkylthio" refers to an alkyl group, as defined above, having a sulfur radical attached thereto. In preferred embodiments, the "alkylthio" moiety is represented by one of -S-alkyl, -S-alkenyl, -S-alkynyl, and -S- $(CH_2)_m-R_8$, wherein m and R_8 are defined above. Representative alkylthio groups include methylthio, ethylthio, and the like.

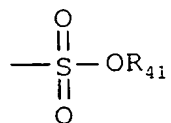
- 20 The term "carbonyl" is art recognized and includes such moieties as can be represented by the general formula:



wherein X is a bond or represents an oxygen or a sulfur, and R₁₁ represents a hydrogen, an alkyl, an alkenyl, -(CH₂)_m-R₈ or a pharmaceutically acceptable salt, R'₁₁ represents a hydrogen, an alkyl, an alkenyl or -(CH₂)_m-R₈, where m and R₈ are as defined above. Where X is an oxygen and R₁₁ or R'₁₁ is not hydrogen, the formula represents an "ester". Where X is an oxygen, and R₁₁ is as defined above, the moiety is referred to herein as a carboxyl group, and particularly when R₁₁ is a hydrogen, the formula represents a "carboxylic acid". Where X is an oxygen, and R'₁₁ is hydrogen, the formula represents a "formate". In general, where the oxygen atom of the above formula is replaced by sulfur, the formula represents a "thiolcarbonyl" group. Where X is a sulfur and R₁₁ or R'₁₁ is not hydrogen, the formula represents a "thiolester." Where X is a sulfur and R₁₁ is hydrogen, the formula represents a "thiolcarboxylic acid." Where X is a sulfur and R'₁₁ is hydrogen, the formula represents a "thiolformate." On the other hand, where X is a bond, and R₁₁ is not hydrogen, the above formula represents a "ketone" group. Where X is a bond, and R₁₁ is hydrogen, the above formula represents an "aldehyde" group.

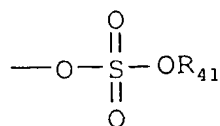
The terms "alkoxyl" or "alkoxy" as used herein refers to an alkyl group, as defined above, having an oxygen radical attached thereto. Representative alkoxyl groups include methoxy, ethoxy, propyloxy, tert-butoxy and the like. An "ether" is two hydrocarbons covalently linked by an oxygen. Accordingly, the substituent of an alkyl that renders that alkyl an ether is or resembles an alkoxyl, such as can be represented by one of -O-alkyl, -O-alkenyl, -O-alkynyl, -O-(CH₂)_m-R₈, where m and R₈ are described above.

The term "sulfonate" is art recognized and includes a moiety that can be represented by the general formula:



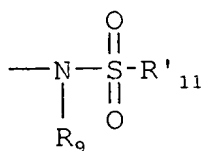
in which R₄₁ is an electron pair, hydrogen, alkyl, cycloalkyl, or aryl.

The term "sulfate" is art recognized and includes a moiety that can be represented by the general formula:



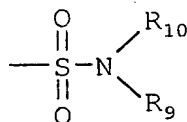
in which R₄₁ is as defined above.

- 5 The term "sulfonamido" is art recognized and includes a moiety that can be represented by the general formula:



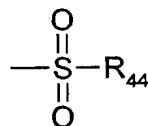
in which R₉ and R'₁₁ are as defined above.

- 10 The term "sulfamoyl" is art-recognized and includes a moiety that can be represented by the general formula:



in which R₉ and R₁₀ are as defined above.

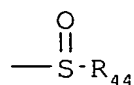
The term "sulfonyl", as used herein, refers to a moiety that can be represented by the general formula:



15

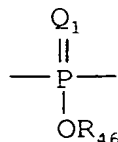
in which R₄₄ is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, or heteroaryl.

The term "sulfoxido" as used herein, refers to a moiety that can be represented by the general formula:



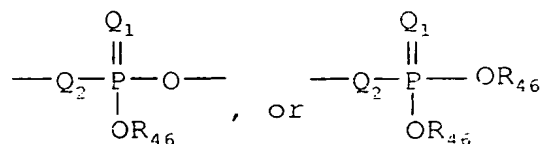
in which R₄₄ is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aralkyl, or aryl.

A "phosphoryl" can in general be represented by the formula:



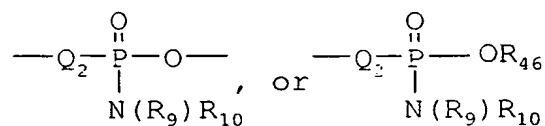
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wherein Q₁ represented S or O, and R₄₆ represents hydrogen, a lower alkyl or an aryl. When used to substitute, e.g., an alkyl, the phosphoryl group of the phosphorylalkyl can be represented by the general formula:



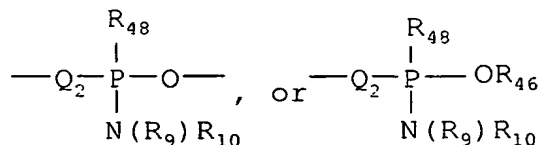
- 10 wherein Q₁ represented S or O, and each R₄₆ independently represents hydrogen, a lower alkyl or an aryl. Q₂ represents O, S or N. When Q₁ is an S, the phosphoryl moiety is a "phosphorothioate".

A "phosphoramidite" can be represented in the general formula:



- 15 wherein R₉ and R₁₀ are as defined above, and Q₂ represents O, S or N.

A "phosphonamidite" can be represented in the general formula:



wherein R₉ and R₁₀ are as defined above, Q₂ represents O, S or N, and R₄₈ represents a lower alkyl or an aryl, Q₂ represents O, S or N.

A "selenoalkyl" refers to an alkyl group having a substituted seleno group attached thereto. Exemplary "selenoethers" which may be substituted on the alkyl are selected from one of -Se-alkyl, -Se-alkenyl, -Se-alkynyl, and -Se-(CH₂)_m-R₇, m and R₇ being defined above.

5 Analogous substitutions can be made to alkenyl and alkynyl groups to produce, for example, aminoalkenyls, aminoalkynyls, amidoalkenyls, amidoalkynyls, iminoalkenyls, iminoalkynyls, thioalkenyls, thioalkynyls, carbonyl-substituted alkenyls or alkynyls.

10 As used herein, the definition of each expression, e.g. alkyl, m, n, etc., when it occurs more than once in any structure, is intended to be independent of its definition elsewhere in the same structure.

15 It will be understood that "substitution" or "substituted with" includes the implicit proviso that such substitution is in accordance with permitted valence of the substituted atom and the substituent, and that the substitution results in a stable compound, e.g., which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, etc.

20 As used herein, the term "substituted" is contemplated to include all permissible substituents of organic compounds. In a broad aspect, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic substituents of organic compounds. Illustrative substituents include, for example, those described herein above. The permissible substituents can be one or more and the same or different for appropriate organic compounds. For purposes of this invention, the heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic compounds described herein which satisfy the valences of the heteroatoms. This
25 invention is not intended to be limited in any manner by the permissible substituents of organic compounds.

30 The phrase "protecting group" as used herein means temporary substituents which protect a potentially reactive functional group from undesired chemical transformations. Examples of such protecting groups include esters of carboxylic

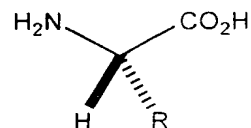
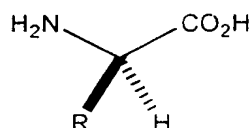
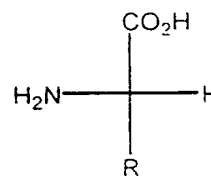
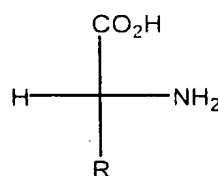
acids, silyl ethers of alcohols, and acetals and ketals of aldehydes and ketones, respectively. The field of protecting group chemistry has been reviewed (Greene, T.W.; Wuts, P.G.M. *Protective Groups in Organic Synthesis*, 2nd ed.; Wiley: New York, 1991).

5 The term "amino acid residue" is known in the art. In general the abbreviations used herein for designating the amino acids and the protective groups are based on recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (see *Biochemistry* (1972) 11:1726-1732). In certain embodiments, the amino acids used in the application of this invention are those naturally occurring
10 amino acids found in proteins, or the naturally occurring anabolic or catabolic products of such amino acids which contain amino and carboxyl groups. Particularly suitable amino acid side chains include side chains selected from those of the following amino acids: glycine, alanine, valine, cysteine, leucine, isoleucine, serine, threonine, methionine, glutamic acid, aspartic acid, glutamine, asparagine, lysine,
15 arginine, proline, histidine, phenylalanine, tyrosine, and tryptophan.

 The term "amino acid residue" further includes analogs, derivatives and congeners of any specific amino acid referred to herein, as well as C-terminal or N-terminal protected amino acid derivatives (e.g. modified with an N-terminal or C-terminal protecting group). For example, the present invention contemplates the use
20 of amino acid analogs wherein a side chain is lengthened or shortened while still providing a carboxyl, amino or other reactive precursor functional group for cyclization, as well as amino acid analogs having variant side chains with appropriate functional groups). For instance, the subject compound can include an amino acid analog such as, for example, cyanoalanine, canavanine, djenkolic acid, norleucine, 3-
25 phosphoserine, homoserine, dihydroxy-phenylalanine, 5-hydroxytryptophan, 1-methylhistidine, 3-methylhistidine, diaminopimelic acid, ornithine, or diaminobutyric acid. Other naturally occurring amino acid metabolites or precursors having side chains which are suitable herein will be recognized by those skilled in the art and are included in the scope of the present invention.

30 Also included are the (D) and (L) stereoisomers of such amino acids when the structure of the amino acid admits of stereoisomeric forms. The configuration of the

amino acids and amino acid residues herein are designated by the appropriate symbols (D), (L) or (DL), furthermore when the configuration is not designated the amino acid or residue can have the configuration (D), (L) or (DL). It will be noted that the structure of some of the compounds of this invention includes asymmetric carbon atoms. It is to be understood accordingly that the isomers arising from such asymmetry are included within the scope of this invention. Such isomers can be obtained in substantially pure form by classical separation techniques and by sterically controlled synthesis. For the purposes of this application, unless expressly noted to the contrary, a named amino acid shall be construed to include both the (D) or (L) stereoisomers. D- and L- α -Amino acids are represented by the following Fischer projections and wedge-and-dash drawings. In the majority of cases, D- and L-amino acids have *R*- and *S*-absolute configurations, respectively.

D- α -amino acidsL- α -amino acids

A "reversed" or "retro" peptide sequence as disclosed herein refers to that part of an overall sequence of covalently-bonded amino acid residues (or analogs or mimetics thereof) wherein the normal carboxyl-to amino direction of peptide bond formation in the amino acid backbone has been reversed such that, reading in the conventional left-to-right direction, the amino portion of the peptide bond precedes (rather than follows) the carbonyl portion. See, generally, Goodman, M. and Chorev, M. Accounts of Chem. Res. 1979, 12, 423.

The reversed orientation peptides described herein include (a) those wherein one or more amino-terminal residues are converted to a reversed ("rev") orientation

(thus yielding a second "carboxyl terminus" at the left-most portion of the molecule), and (b) those wherein one or more carboxyl-terminal residues are converted to a reversed ("rev") orientation (yielding a second "amino terminus" at the right-most portion of the molecule). A peptide (amide) bond cannot be formed at the interface
5 between a normal orientation residue and a reverse orientation residue.

Therefore, certain reversed peptide compounds of the invention can be formed by utilizing an appropriate amino acid mimetic moiety to link the two adjacent portions of the sequences depicted above utilizing a reversed peptide (reversed amide) bond. In case (a) above, a central residue of a diketo compound may conveniently be
10 utilized to link structures with two amide bonds to achieve a peptidomimetic structure. In case (b) above, a central residue of a diamino compound will likewise be useful to link structures with two amide bonds to form a peptidomimetic structure.

The reversed direction of bonding in such compounds will generally, in addition, require inversion of the enantiomeric configuration of the reversed amino
15 acid residues in order to maintain a spatial orientation of side chains that is similar to that of the non-reversed peptide. The configuration of amino acids in the reversed portion of the peptides is preferably (D), and the configuration of the non-reversed portion is preferably (L). Opposite or mixed configurations are acceptable when appropriate to optimize a binding activity.

20 Certain compounds of the present invention may exist in particular geometric or stereoisomeric forms. The present invention contemplates all such compounds, including *cis*- and *trans*-isomers, *R*- and *S*-enantiomers, diastereomers, (D)-isomers, (L)-isomers, the racemic mixtures thereof, and other mixtures thereof, as falling within the scope of the invention. Additional asymmetric carbon atoms may be
25 present in a substituent such as an alkyl group. All such isomers, as well as mixtures thereof, are intended to be included in this invention.

If, for instance, a particular enantiomer of a compound of the present invention is desired, it may be prepared by asymmetric synthesis, or by derivation with a chiral auxiliary, where the resulting diastereomeric mixture is separated and the auxiliary
30 group cleaved to provide the pure desired enantiomers. Alternatively, where the molecule contains a basic functional group, such as amino, or an acidic functional

group, such as carboxyl, diastereomeric salts are formed with an appropriate optically-active acid or base, followed by resolution of the diastereomers thus formed by fractional crystallization or chromatographic means well known in the art, and subsequent recovery of the pure enantiomers.

5 Contemplated equivalents of the compounds described above include compounds which otherwise correspond thereto, and which have the same general properties thereof (e.g. the ability to bind to opioid receptors), wherein one or more simple variations of substituents are made which do not adversely affect the efficacy of the compound in binding to opioid receptors. In general, the compounds of the
10 present invention may be prepared by the methods illustrated in the general reaction schemes as, for example, described below, or by modifications thereof, using readily available starting materials, reagents and conventional synthesis procedures. Thus, the contemplated equivalents include small molecule inhibitors of the E1-E2 infections which, e.g., bind either E1 or E2. In these reactions, it is also possible to
15 make use of variants which are in themselves known, but are not mentioned here.

For purposes of this invention, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, Handbook of Chemistry and Physics, 67th Ed., 1986-87, inside cover. Also for purposes of this invention, the term "hydrocarbon" is contemplated to include all permissible
20 compounds having at least one hydrogen and one carbon atom. In a broad aspect, the permissible hydrocarbons include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic organic compounds which can be substituted or unsubstituted.

As used herein, the term "transfection" means the introduction of a nucleic
25 acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with
30 which they are operably linked.

Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of the subject peptide. Accordingly, the term transcriptional regulatory
5 sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel: *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990).

The term "gene construct" refers to a vector, plasmid, viral genome or the like which includes a coding sequence, can transfect cells, preferably mammalian cells,
10 and can cause expression of the E2 for an E2 peptide of the cells transfected with the construct. The term "gene construct" does not include a wild-type papillomavirus genome, and preferably does not include expressible coding sequences for one or more of a papillomavirus E6 or E7 proteins.

As used herein, the term "pharmaceutically acceptable" refers to a carrier
15 medium which does not interfere with the effectiveness of the biological activity of the active ingredients and which is not excessively toxic to the hosts of the concentrations of which it is administered. The administration(s) may take place by any suitable technique, including subcutaneous and parenteral administration, preferably parenteral. Examples of parenteral administration include intravenous,
20 intraarterial, intramuscular, and intraperitoneal, with intravenous being preferred.

As used herein, the term "prophylactic or therapeutic" treatment refers to administration to the host of the papillomavirus medicament. If it is administered prior to exposure to the virus, the treatment is prophylactic (i.e., it protects the host against infection), whereas if administered after infection or initiation of the disease, the
25 treatment is therapeutic (i.e., it combats the existing infection or cancer).

As used herein the term " papillomavirus disease" refers to any kind of infection or disorder caused by the virus, including cancers and warts. Thus, the term includes symptoms and side effect of any papillomavirus infection, including latent, persistent and sub-clinical infections, whether or not the infection is clinically
30 apparent.

The term "cell-proliferative disorder" denotes malignant as well as nonmalignant cell populations which morphologically often appear to differ from the surrounding tissue.

5 *III. Methods of Treatment*

Diseases which may be prevented and/or treated by the processes and compositions of this invention are those caused by the etiological agent, papillomavirus, and may be the result of clinical or sub-clinical PV infections. Such diseases include, for example, epithelial malignancies, anogenital malignancies, such
10 as cervical cancer, malignant lesions, benign lesions, papillomacarcinomas, papilloadenocystomas, papilloma neurophathicum, papillomatosis, cutaneous and mucosal papillomas, condylomas, oral, pharyngeal, laryngeal, and tongue papillomas, fibroblastic tumors and other pathological conditions associated with papillomavirus. The E2 inhibitors of this invention may also be used to treat epithelial and internal
15 fibropapillomas in animals.

In addition, as described above, a wide variety of warts are found on human skin and are caused by the human papillomavirus (HPV). For example, the following types of warts are found on human skin and are caused by the human papillomavirus (HPV): common warts (*verruca vulgaris*), plantar warts, palmar warts, planar warts
20 (*verruca plana*), mosaic warts, and venereal warts (*condyloma acuminatum*). These skin growths are unsightly, irritating, and potentially oncogenic (carcinogenic), and their removal is desired.

Genital warts, also referred to as venereal warts and condylomata acuminata, are one of the most serious manifestations of HPV infection. As reported by the
25 Center for Disease Control, the sexual mode of transmission of genital warts is well established and the incidence of genital warts is on the increase. The seriousness of genital warts is underlined by the finding that HPV DNA can be found in all grades of cervical intraepithelial neoplasia (CIN I-III) and that a specific subset of HPV types can be found in carcinoma *in situ* of the cervix. Consequently, women with genital
30 warts, containing specific HPV types are now considered at high risk for the

development of cervical cancer. Current treatments for genital warts are inadequate. According to the present invention, a method of treating a patient having one or more genital warts comprises the administration of a pharmaceutical composition including an E2 peptidomimetic, or a gene construct encoding an E2 peptide including E39, so as to inhibit growth of the wart. In preferred embodiments, the wart(s), or other PV-containing cells, are contacted directly with the pharmaceutical composition. The subject method can be used to treat, e.g., condyloma acuminata and/or flat cervical warts.

Laryngeal papillomas are benign epithelial tumors of the larynx. Two PV types, HPV-6 and HPV-11, are most commonly associated with laryngeal papillomas. According to the method of the present invention, laryngeal papillomas are treated administering a pharmaceutical composition including the therapeutic E2 peptidomimetic, or a gene construct encoding an E2-derived peptide, so as to inhibit growth of the papillomas.

The most common disease associated with papillomavirus infection are benign skin warts. Common warts generally contain HPV types 1, 2, 3, 4 or 10. These warts typically occur on the soles of feet, plantar warts, or on the hands. Common skin warts are most often found in children and young adults. Later in life the incidence of common warts decreases presumably due to immunologic and physiologic changes. Plantar warts can often be debilitating and require surgical removal and they frequently reoccur after surgery. As above, patients suffering from common warts can be treated by the administration of an effective amount of an E2 peptidomimetic according to the present invention, or a gene therapy construct which encodes the therapeutic E2 peptide. In preferred embodiments, the peptide or gene construct are applied, in the appropriate formulations, directly to the area of the skin afflicted with the wart(s). Similar methods and compositions may be useful in the treatment of epidermodysplasia verruciformis (EV), a rare genetically transmitted disease which is characterized by disseminated flat warts that appear as small reddish macules.

In addition, the subject method and compositions may be used to treat lesions resulting from cellular transformation for which HPV is an etiological agent, e.g., in the treatment of cervical cancer.

IV. Exemplary Drug Screening Assays

A variety of drug screening techniques can be readily adapted to the E1-E2 interaction in order to provide high throughput screening of peptide, peptidomimetic
5 or other small molecule libraries. Such assays can be used to optimize a lead compound, or to assess the potential PV inhibitory effect of a test compound.

In one embodiment, simple competition assays can be used to assess the ability of a test compound to disrupt the interaction of an E1-E2 complex. In other
embodiments, cell-based assays which detect PV replication or Papilloma virus
10 replication can be used to assess the biological activity of a test compound.

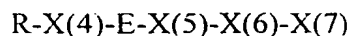
V. Exemplary Compositions

In certain embodiments of the present invention, such as for topical administration to the epidermis, the subject E2 pharmaceutical can be a peptide, e.g.,
15 having a naturally occurring peptide backbone and amino acid side chains, though it may be N-terminally and/or C-terminally protected.

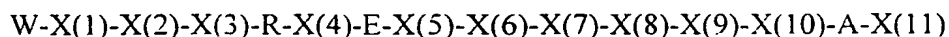
In preferred embodiments, the peptidyl component of the subject compounds includes, in addition to the core E2 sequences, as described herein, no more than about 25 amino acid residues of a protein in which an E2 motif naturally exists, more
20 preferably no more than 10-15, and even more preferably 6 or less. With the exception of certain chimeric E2 compositions described herein, such as fusion proteins, a preferred composition (especially for ectopic application) includes a peptide comprising an E2 core motif and having a molecular weight in the range of about 1500 to 7500 daltons, more preferably from about 2000 to 5000 daltons, and even
25 more preferably in the range of about 2000 to 2750 daltons. The peptide, in addition to the E2 core motif, may include other amino acid residues, such as a transcytosis peptide, and may be derivatized at one or more backbone or sidechain points with, e.g. peptides, nucleic acids, carbohydrates, etc. In certain embodiments, the peptide is derivatized with one or more functional groups that enhance cellular uptake and/or
30 impair the half-life of the E2 core motif.

This invention further contemplates a method of generating sets of combinatorial libraries of the subject E2 peptides which is especially useful for identifying potential variant sequences (e.g. homologs) that are functional in inhibiting E1-E2 interactions. Combinatorially-derived homologs can be generated
5 which have, e.g., greater affinity, an enhanced potency relative to native E2 peptide sequences, or intracellular half-lives different than the corresponding wild-type E2 peptide. For example, the altered peptide can be rendered either more stable or less stable to proteolytic degradation or other cellular process which result in destruction of, or otherwise inactivation of, the peptide. Such homologs can be utilized to alter
10 the envelope of therapeutic application by modulating the half-life of the peptide. For instance, a short half-life can give rise to more transient biological effects and can allow tighter control of peptide levels within the cell.

In a representative embodiment of this method, the amino acid sequences for a population of E2 motifs are aligned, preferably to promote the highest homology
15 possible. Such a population of variants can include, for example, E2 peptides from both high and low risk human papillomaviruses, as well as PV from other animals. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. To illustrate, as set out above inspection of E2 sequences reveals a consensus sequence (see Figures 5A and 5B).
20 Similarly, Figure 12 reveals a shorter consensus sequence based upon critical binding residues. Based on these alignments, combinatorial libraries can be generated from this portion of E2 peptides so as to have an amino acid sequence that includes an E2 core sequence represented by the formula:



25 and wherein this six sequence formula may be elongated e.g., to obtain peptides up to and including the fifteen sequence represented by the formula:



wherein each of Xaa(1)-Xaa(11) are selected from one of the amino acid residues of the same position in Figure 5B. Peptides larger than the 15-mer core are, of course,
30 also contemplated.

Further expansion of the combinatorial library can be made, for example, by including amino acids which would represent conservative mutations at one or more of the degenerate positions. Inclusion of such conservative mutations can give rise to a library of potential E2 peptide sequences represented by the above formula, but
5 wherein Xaa(1) can be an amino acid residue having a polar sidechain, such as arg, asn, asp, cys, glu, gln, his, lys, ser, thr or tyr, as set out by the core structures above. Alternatively, amino acid replacement at degenerate positions can be based on steric criteria, e.g. isosteric replacement, without regard for polarity or charge of amino acid sidechains. Similarly, completely random mutagenesis of one or more of the variant
10 positions (Xaa) can be carried out, e.g., each of Xaa(1)-(11) can be any of the 20 amino acids (or other analogs thereof).

In one embodiment the E2 peptide library can be derived by combinatorial chemistry, such as by techniques which are available in the art for generating combinatorial libraries of small organic/peptide libraries. See, for example, Blondelle
15 et al. (1995) *Trends Anal. Chem.* 14:83; the Affymax U.S. Patents 5,359,115 and 5,362,899; the Ellman U.S. Patent 5,288,514; the Still et al. PCT publication WO 94/08051; Chen et al. (1994) *JACS* 116:2661; Kerr et al. (1993) *JACS* 115:252; PCT publications WO92/10092, WO93/09668 and WO91/07087; and the Lerner et al. PCT publication WO93/20242).

20 In a preferred embodiment, the combinatorial peptide library is produced by way of a degenerate library of genes encoding a library of polypeptides which each include at least a portion of potential E2 sequences. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential E2 nucleotide sequences are expressible as individual
25 polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display) containing the set of E2 peptide sequences therein.

There are many ways by which the gene library of potential E2 homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and
30 the synthetic genes then be ligated into an appropriate gene for expression. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences

encoding the desired set of potential E2 sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp. 273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of E2 sequences. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Such illustrative assays are amenable to high throughput analysis as necessary to screen large numbers of degenerate sequences created by combinatorial mutagenesis techniques.

In an illustrative embodiment of a screening assay, the E2 gene library can be expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at very high concentrations, a large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E.coli* filamentous phages M13, fd, and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat

proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) *J. Biol. Chem.* 267:16007-16010; Griffiths et al. (1993) *EMBO J* 12:725-734; Clackson et al. (1991) *Nature* 5 352:624-628; and Barbas et al. (1992) *PNAS* 89:4457-4461).

For example, the recombinant phage antibody system (RPAS, Pharmacia Catalog number 27-9400-01) can be easily modified for use in expressing and screening E2 motif combinatorial libraries of the present invention. For instance, the pCANTAB 5 phagemid of the RPAS kit contains the gene which encodes the phage
10 gIII coat protein. The E2 combinatorial gene library can be cloned into the phagemid adjacent to the gIII signal sequence such that it will be expressed as a gIII fusion protein. After ligation, the phagemid is used to transform competent *E. coli* TG1 cells. Transformed cells are subsequently infected with M13KO7 helper phage to rescue the phagemid and its candidate E2 gene insert. The resulting recombinant
15 phage contain phagemid DNA encoding a specific candidate E2 peptide, and display one or more copies of the corresponding fusion coat protein. The phage-displayed candidate proteins which are capable of, for example, binding an E1 protein, are selected or enriched by panning. For instance, the phage library can be panned on glutathione immobilized E1-GST fusion proteins, and unbound phage washed away
20 from the cells. The bound phage is then isolated, and if the recombinant phage express at least one copy of the wild type gIII coat protein, they will retain their ability to infect *E. coli*. Thus, successive rounds of reinfection of *E. coli*, and panning will greatly enrich for E2 homologs which can then be screened for further PV inhibitory activities. Subsequent selection, e.g. of a reduced set of variants from the
25 library, may then be based upon more meaningful criteria rather than simple E1-binding ability. For instance, intracellular half-life or selectivity can become selection criteria in secondary screens.

Combined with certain formulations, such peptides can be effective intracellular agents for, e.g., inhibiting PV infection of epithelial cells. However, in
30 order to increase the efficacy of such peptides, the E2 peptide can be provided a fusion peptide along with a second peptide which promotes "transcytosis", e.g.,

uptake of the peptide by epithelial cells. To illustrate, the E2 peptide of the present invention can be provided as part of a fusion polypeptide with all or a fragment of the N-terminal domain of the HIV protein Tat, e.g., residues 1-72 of Tat or a smaller fragment thereof which can promote transcytosis. In other embodiments, the E2 peptide can be provided a fusion polypeptide with all or a portion of the antennapedia III protein.

To further illustrate, the E2 peptide (or peptidomimetic) can be provided as a chimeric peptide which includes a heterologous peptide sequence ("internalizing peptide") which drives the translocation of an extracellular form of a E2 peptide sequence across a cell membrane in order to facilitate intracellular localization of the E2 peptide. In this regard, the therapeutic E2 sequence is one which is active intracellularly. The internalizing peptide, by itself, is capable of crossing a cellular membrane by, e.g., transcytosis, at a relatively high rate. The internalizing peptide is conjugated, e.g., as a fusion protein, to the E2 peptide. The resulting chimeric peptide is transported into cells at a higher rate relative to the activator polypeptide alone to thereby provide an means for enhancing its introduction into cells to which it is applied, e.g., to enhance topical applications of the E2 peptide.

In one embodiment, the internalizing peptide is derived from the *Drosophila* antennapedia protein, or homologs thereof. The 60 amino acid long long homeodomain of the homeo-protein antennapedia has been demonstrated to translocate through biological membranes and can facilitate the translocation of heterologous polypeptides to which it is couples. See for example Derossi et al. (1994) J Biol Chem 269:10444-10450; and Perez et al. (1992) J Cell Sci 102:717-722. Recently, it has been demonstrated that fragments as small as 16 amino acids long of this protein are sufficient to drive internalization. See Derossi et al. (1996) J Biol Chem 271:18188-18193.

The present invention contemplates an E2 peptide or peptidomimetic sequence as described herein, and at least a portion of the Antennapedia protein (or homolog thereof) sufficient to increase the transmembrane transport of the chimeric protein, relative to the E2 peptide or peptidomimetic, by a statistically significant amount. As discussed in the Exemplification, fusion peptides were tested for their ability to inhibit

HPV-16 E1-E2 interaction *in vitro*. Figure 14 depicts the inhibition of Ant-WP peptide at levels comparable to that of wild-type peptide. The similar inhibition of the E39A counterpart is also contemplated.

Another example of an internalizing peptide is the HIV transactivator (TAT) protein. This protein appears to be divided into four domains (Kuppuswamy et al. (1989) Nucl. Acids Res. 17:3551-3561). Purified TAT protein is taken up by cells in tissue culture (Frankel and Pabo. (1989) Cell 55:1189-1193), and peptides, such as the fragment corresponding to residues 37 -62 of TAT, are rapidly taken up by cell *in vitro* (Green and Loewenstein. (1989) Cell 55:1179-1188). The highly basic region mediates internalization and targeting of the internalizing moiety to the nucleus (Ruben et al., (1989) J. Virol. 63:1-8).

Another exemplary transcellular polypeptide can be generated to include a sufficient portion of mastoparan (T. Higashijima et al., (1990) J. Biol. Chem. 265:14176) to increase the transmembrane transport of the chimeric protein.

While not wishing to be bound by any particular theory, it is noted that hydrophilic polypeptides may be also be physiologically transported across the membrane barriers by coupling or conjugating the polypeptide to a transportable peptide which is capable of crossing the membrane by receptor-mediated transcytosis. Suitable internalizing peptides of this type can be generated using all or a portion of, e.g., a histone, insulin, transferrin, basic albumin, prolactin and insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II) or other growth factors. For instance, it has been found that an insulin fragment, showing affinity for the insulin receptor on capillary cells, and being less effective than insulin in blood sugar reduction, is capable of transmembrane transport by receptor-mediated transcytosis and can therefor serve as an internalizing peptide for the subject transcellular peptides and peptidomimetics. Preferred growth factor-derived internalizing peptides include EGF (epidermal growth factor)-derived peptides, such as CMHIESLDSYTC and CMYIEALDKYAC; TGF- beta (transforming growth factor beta)-derived peptides; peptides derived from PDGF (platelet-derived growth factor) or PDGF-2; peptides derived from IGF-I (insulin-like growth factor) or IGF-II; and FGF (fibroblast growth factor)-derived peptides.

Another class of translocating/internalizing peptides exhibits pH-dependent membrane binding. For an internalizing peptide that assumes a helical conformation at an acidic pH, the internalizing peptide acquires the property of amphiphilicity. e.g., it has both hydrophobic and hydrophilic interfaces. More specifically, within a pH range of approximately 5.0-5.5, an internalizing peptide forms an alpha-helical, amphiphilic structure that facilitates insertion of the moiety into a target membrane. An alpha-helix-inducing acidic pH environment may be found, for example, in the low pH environment present within cellular endosomes. Such internalizing peptides can be used to facilitate transport of E2 peptides and peptidomimetics, taken up by an endocytic mechanism, from endosomal compartments to the cytoplasm.

A preferred pH-dependent membrane-binding internalizing peptide includes a high percentage of helix-forming residues, such as glutamate, methionine, alanine and leucine. In addition, a preferred internalizing peptide sequence includes ionizable residues having pKa's within the range of pH 5-7, so that a sufficient uncharged membrane-binding domain will be present within the peptide at pH 5 to allow insertion into the target cell membrane.

A particularly preferred pH-dependent membrane-binding internalizing peptide in this regard is aa1-aa2-aa3-EAALA(EALA)4-EALEALAA-amide, which represents a modification of the peptide sequence of Subbarao et al. (Biochemistry 26:2964, 1987). Within this peptide sequence, the first amino acid residue (aa1) is preferably a unique residue, such as cysteine or lysine, that facilitates chemical conjugation of the internalizing peptide to a targeting protein conjugate. Amino acid residues 2-3 may be selected to modulate the affinity of the internalizing peptide for different membranes. For instance, if both residues 2 and 3 are lys or arg, the internalizing peptide will have the capacity to bind to membranes or patches of lipids having a negative surface charge. If residues 2-3 are neutral amino acids, the internalizing peptide will insert into neutral membranes.

Yet other preferred internalizing peptides include peptides of apo-lipoprotein A-1 and B; peptide toxins, such as melittin, bombolittin, delta hemolysin and the pardaxins; antibiotic peptides, such as alamethicin; peptide hormones, such as calcitonin, corticotrophin releasing factor, beta endorphin, glucagon, parathyroid

hormone, pancreatic polypeptide; and peptides corresponding to signal sequences of numerous secreted proteins. In addition, exemplary internalizing peptides may be modified through attachment of substituents that enhance the alpha-helical character of the internalizing peptide at acidic pH.

5 Yet another class of internalizing peptides suitable for use within the present invention include hydrophobic domains that are "hidden" at physiological pH, but are exposed in the low pH environment of the target cell endosome. Upon pH-induced unfolding and exposure of the hydrophobic domain, the moiety binds to lipid bilayers and effects translocation of the covalently linked polypeptide into the cell cytoplasm.

10 Such internalizing peptides may be modeled after sequences identified in, e.g., *Pseudomonas* exotoxin A, clathrin, or Diphtheria toxin.

Pore-forming proteins or peptides may also serve as internalizing peptides herein. Pore-forming proteins or peptides may be obtained or derived from, for example, C9 complement protein, cytolytic T-cell molecules or NK-cell molecules.

15 These moieties are capable of forming ring-like structures in membranes, thereby allowing transport of attached polypeptide through the membrane and into the cell interior.

Mere membrane intercalation of an internalizing peptide may be sufficient for translocation of the E2 peptide or peptidomimetic, across cell membranes. However,

20 translocation may be improved by attaching to the internalizing peptide a substrate for intracellular enzymes (i.e., an "accessory peptide"). It is preferred that an accessory peptide be attached to a portion(s) of the internalizing peptide that protrudes through the cell membrane to the cytoplasmic face. The accessory peptide may be advantageously attached to one terminus of a translocating/internalizing moiety or

25 anchoring peptide. An accessory moiety of the present invention may contain one or more amino acid residues. In one embodiment, an accessory moiety may provide a substrate for cellular phosphorylation (for instance, the accessory peptide may contain a tyrosine residue).

An exemplary accessory moiety in this regard would be a peptide substrate for

30 N-myristoyl transferase, such as GNAAAARR (Eubanks et al., in: Peptides, Chemistry and Biology, Garland Marshall (ed.), ESCOM, Leiden, 1988, pp. 566-69)

In this construct, an internalizing peptide would be attached to the C-terminus of the accessory peptide, since the N-terminal glycine is critical for the accessory moiety's activity. This hybrid peptide, upon attachment to an E2 peptide or peptidomimetic at its C-terminus, is N-myristylated and further anchored to the target cell membrane, e.g., it serves to increase the local concentration of the peptide at the cell membrane.

To further illustrate use of an accessory peptide, a phosphorylatable accessory peptide is first covalently attached to the C-terminus of an internalizing peptide and then incorporated into a fusion protein with an E2 peptide or peptidomimetic. The peptide component of the fusion protein intercalates into the target cell plasma membrane and, as a result, the accessory peptide is translocated across the membrane and protrudes into the cytoplasm of the target cell. On the cytoplasmic side of the plasma membrane, the accessory peptide is phosphorylated by cellular kinases at neutral pH. Once phosphorylated, the accessory peptide acts to irreversibly anchor the fusion protein into the membrane. Localization to the cell surface membrane can enhance the translocation of the polypeptide into the cell cytoplasm.

Suitable accessory peptides include peptides that are kinase substrates, peptides that possess a single positive charge, and peptides that contain sequences which are glycosylated by membrane-bound glycotransferases. Accessory peptides that are glycosylated by membrane-bound glycotransferases may include the sequence x-NLT-x, where "x" may be another peptide, an amino acid, coupling agent or hydrophobic molecule, for example. When this hydrophobic tripeptide is incubated with microsomal vesicles, it crosses vesicular membranes, is glycosylated on the luminal side, and is entrapped within the vesicles due to its hydrophilicity (C. Hirschberg et al., (1987) Ann. Rev. Biochem. 56:63-87). Accessory peptides that contain the sequence x-NLT-x thus will enhance target cell retention of corresponding polypeptide.

In another embodiment of this aspect of the invention, an accessory peptide can be used to enhance interaction of the E2 peptide or peptidomimetic with the target cell. Exemplary accessory peptides in this regard include peptides derived from cell adhesion proteins containing the sequence "RGD", or peptides derived from laminin containing the sequence CDPGYIGSRC. Extracellular matrix glycoproteins, such as

fibronectin and laminin, bind to cell surfaces through receptor-mediated processes. A tripeptide sequence, RGD, has been identified as necessary for binding to cell surface receptors. This sequence is present in fibronectin, vitronectin, C3bi of complement, von-Willebrand factor, EGF receptor, transforming growth factor beta, collagen type I, lambda receptor of E. Coli, fibrinogen and Sindbis coat protein (E. Ruoslahti, Ann. Rev. Biochem. 57:375-413, 1988). Cell surface receptors that recognize RGD sequences have been grouped into a superfamily of related proteins designated "integrins". Binding of "RGD peptides" to cell surface integrins will promote cell-surface retention, and ultimately translocation, of the polypeptide.

As described above, the internalizing and accessory peptides can each, independently, be added to E2 peptide or peptidomimetic by either chemical cross-linking or in the form of a fusion protein. In the instance of fusion proteins, unstructured polypeptide linkers can be included between each of the peptide moieties.

In general, the internalization peptide will be sufficient to also direct export of the polypeptide. However, where an accessory peptide is provided, such as an RGD sequence, it may be necessary to include a secretion signal sequence to direct export of the fusion protein from its host cell. In preferred embodiments, the secretion signal sequence is located at the extreme N-terminus, and is (optionally) flanked by a proteolytic site between the secretion signal and the rest of the fusion protein.

In an exemplary embodiment, an E2 peptide or peptidomimetic is engineered to include an integrin-binding RGD peptide/SV40 nuclear localization signal (see, for example Hart SL et al., 1994; J. Biol. Chem. 269:12468-12474), such as encoded by the nucleotide sequence provided in the NdeI-EcoRI fragment:

catatgggtggctgccgtggcgatatgttcggttgccgtgctcctccaaaaagaagagaaag-gtagctggattc,

which encodes the RGD/SV40 nucleotide sequence: MGGCRGDMFGCGAPP-KKKRKVAGF. In another embodiment, the protein can be engineered with the HIV-1 tat(1-72) polypeptide, e.g., as provided by the NdeI-EcoRI fragment: catatggagccagtagatcctagactagagccc-

tggaagcatccaggaagtcagcctaaaactgcttgtagcaattgctattgtaaaaagtgttgcttcattgccaagttgtttcat

aacaaaagcccttggcatctcctatggcaggaagaagcggagacagcgacgaagacctcctcaaggcagtcagactcat

caagtttctctaagtaagcaaggattc, which encodes the HIV-1 tat(1-72) peptide sequence:
MEPVDPRLEPWKHPGSQPKT-

ACTNCYCKKCCFHCQVCFITKALGISYGRKKRRRQRRRPPQGSQTHQVSLSKQ.

In still another embodiment, the fusion protein includes the HSV-1 VP22 polypeptide
5 (Elliott G., O'Hare P (1997) Cell, 88:223-233) provided by the Nde1-EcoR1
fragment:

cat atg acc tct cgc cgc tcc gtg aag tcg ggt ccg cgg gag gtt ccg cgc gat gag tac gag gat
ctg tac tac acc ccg tct tca ggt atg gcg agt ccc gat agt ccg cct gac acc tcc cgc cgt ggc
gcc cta cag aca cgc tcg cgc cag agg ggc gag gtc cgt ttc gtc cag tac gac gag tcg gat tat
10 gcc ctg tac ggg ggc tcg tca tcc gaa gac gac gaa cac ccg gag gtc ccc cgg acg cgg cgt ccc
gtt tcc ggg gcg gtt ttg tcc ggc ccg ggg cct gcg cgg gcg cct ccg cca ccc gct ggg tcc gga
ggg gcc gga cgc aca ccc acc acc gcc ccc cgg gcc ccc cga acc cag cgg gtg gcg act aag
gcc ccc gcg gcc ccg gcg gcg gag acc acc cgc ggc agg aaa tcg gcc cag cca gaa tcc gcc
gca ctg cca gac gcc ccc gcg tcg acg gcg cca acc cga tcc aag aca ccc gcg cag ggg ctg
15 gcc aga aag ctg cac ttg agc acc gcc ccc cca aac ccc gac gcg cca tgg acc ccc cgg gtg gcc
ggc ttg aac aag cgc gtc ttc tgc gcc gcg gtc ggg cgc ctg gcg gcc atg cat gcc cgg atg gcg
gcg gtc cag ctg tgg gac atg tcg cgt ccg cgc aca gac gaa gac ctg aac gaa ctg ctt ggc atc
acc acc atc cgc gtg acg gtc tgc gag ggc aaa aac ctg ctt cag cgc gcc aac gag ttg gtg aat
cca gac gtg gtg cag gac gtc gac gcg gcc acg gcg act cga ggg cgt tct gcg gcg tcg cgc ccc
20 acc gag cga cct cga gcc cca gcc cgc tcc gct tct cgc ccc aga cgg ccc gtc gag gaa ttc

which encodes the HSV-1 VP22 peptide having the sequence:

MTSRRSVKSGPREVPRDEYEDLYYTPSSGMASPDSPDTSRRGALQTR
SRQRGEVRFVQYDESDYALYGGSSSEDDEHPEVPRTRRPVSGAVLSGPGPAR
APPPAGSGGAGRTPTTAPRAPRTGRVATKAPAAPAAETTRGRKSAQPESAA
25 LPDAPASTAPTRSKTPAQGLARKLHFSTAPPNPDAPWTPRVAGFNKRVFCAA
VGRLAAMHARMAAVQLWDMSRPRTDEDLNELLGITTIRVTVCEGKNLLQRA
NELVNPDVVQDVDAATATRGRSAASRPTERPRAPARSASRPRRPVE

In still another embodiment, the fusion protein includes the C-terminal domain
of the VP22 protein from, e.g., the nucleotide sequence (Nde1-EcoR1 fragment):

cat atg gac gtc gac gcg gcc acg gcg act cga ggg cgt tct gcg gcg tcg cgc ccc acc gag cga
cct cga gcc cca gcc cgc tcc gct tct cgc ccc aga cgg ccc gtc gag gaa ttc

which encodes the VP22 (C-terminal domain) peptide sequence:
MDVDAATATRGRSA-ASRPTERPRAPARSASRPRRPVE

- 5 In certain instances, it may also be desirable to include a nuclear localization signal as part of the E2 peptide.

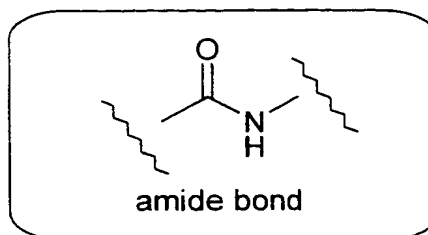
 In the generation of fusion polypeptides including the subject E2 peptides, it may be necessary to include unstructured linkers in order to ensure proper folding of the various peptide domains, and prevent steric or other interference of the
10 heterologous domains with the PV inhibitory activity of the E2 peptide. Many synthetic and natural linkers are known in the art and can be adapted for use in the present invention, including the (Gly₃Ser)₄ linker.

 In other embodiments, the subject E2 therapeutics are peptidomimetics of the E2 peptide. Peptidomimetics are compounds based on, or derived from, peptides and
15 proteins. The E2 peptidomimetics of the present invention typically can be obtained by structural modification of a known E2 peptide sequence using unnatural amino acids, conformational restraints, isosteric replacement, and the like. The subject peptidomimetics constitute the continuum of structural space between peptides and non-peptide synthetic structures; E2 peptidomimetics may be useful, therefore, in
20 delineating pharmacophores and in helping to translate peptides into nonpeptide compounds with the activity of the parent E2 peptides.

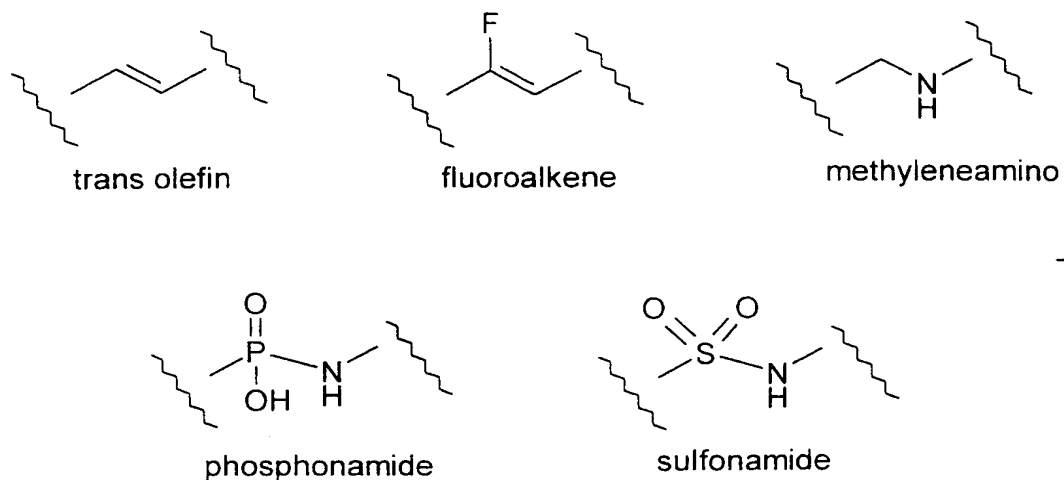
 Moreover, as is apparent from the present disclosure, mimetopes of the subject E2 peptides can be provided. Such peptidomimetics can have such attributes as being non-hydrolyzable (e.g., increased stability against proteases or other physiological
25 conditions which degrade the corresponding peptide), increased specificity and/or potency for inhibition of PV replication, and increased cell permeability for intracellular localization of the peptidomimetic. For illustrative purposes, peptide analogs of the present invention can be generated using, for example, benzodiazepines (e.g., see Freidinger et al. in Peptides: Chemistry and Biology, G.R. Marshall ed.,
30 ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings

(Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands. 1988. p123), C-7 mimics (Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988. p. 105), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL. 1985), β -turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans 1*:1231), β -aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71), diaminoketones (Natarajan et al. (1984) *Biochem Biophys Res Commun* 124:141), and methyleneamino-modified (Roark et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands. 1988, p134). Also, see generally, Session III: Analytic and synthetic methods, in in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988)

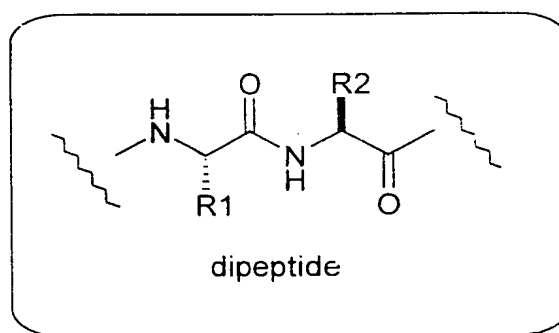
In addition to a variety of sidechain replacements which can be carried out to generate the subject E2 peptidomimetics, the present invention specifically contemplates the use of conformationally restrained mimics of peptide secondary structure. Numerous surrogates have been developed for the amide bond of peptides. Frequently exploited surrogates for the amide bond include the following groups (i) trans-olefins, (ii) fluoroalkene, (iii) methyleneamino, (iv) phosphonamides, and (v) sulfonamides.



Examples of Surrogates

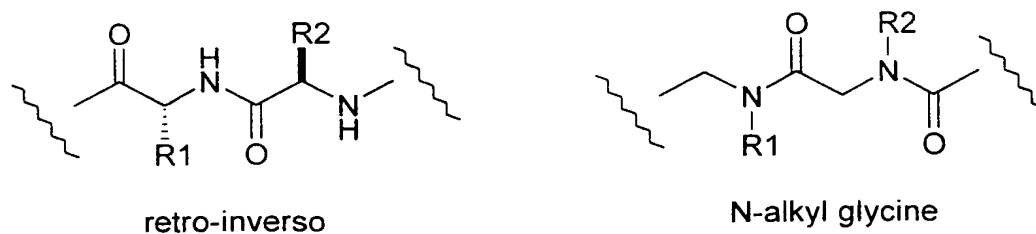


Additionally, peptidomimetics based on more substantial modifications of the backbone of the E2 peptide can be used. Peptidomimetics which fall in this category include (i) retro-inverso analogs, and (ii) N-alkyl glycine analogs (so-called peptoids).



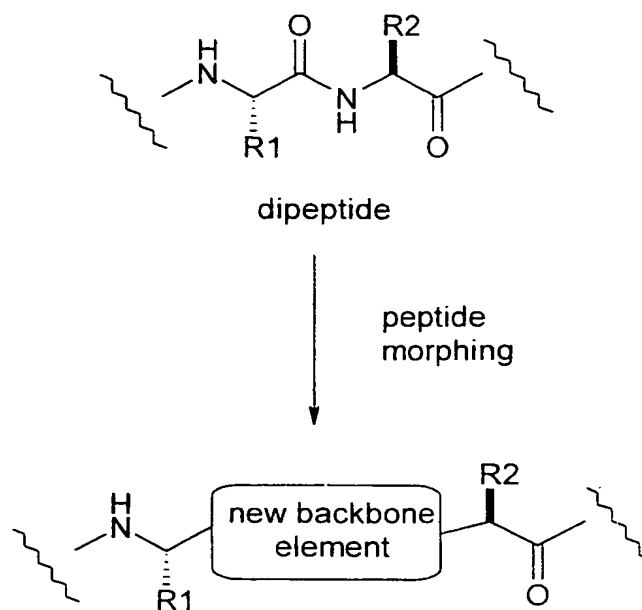
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Examples of analogs

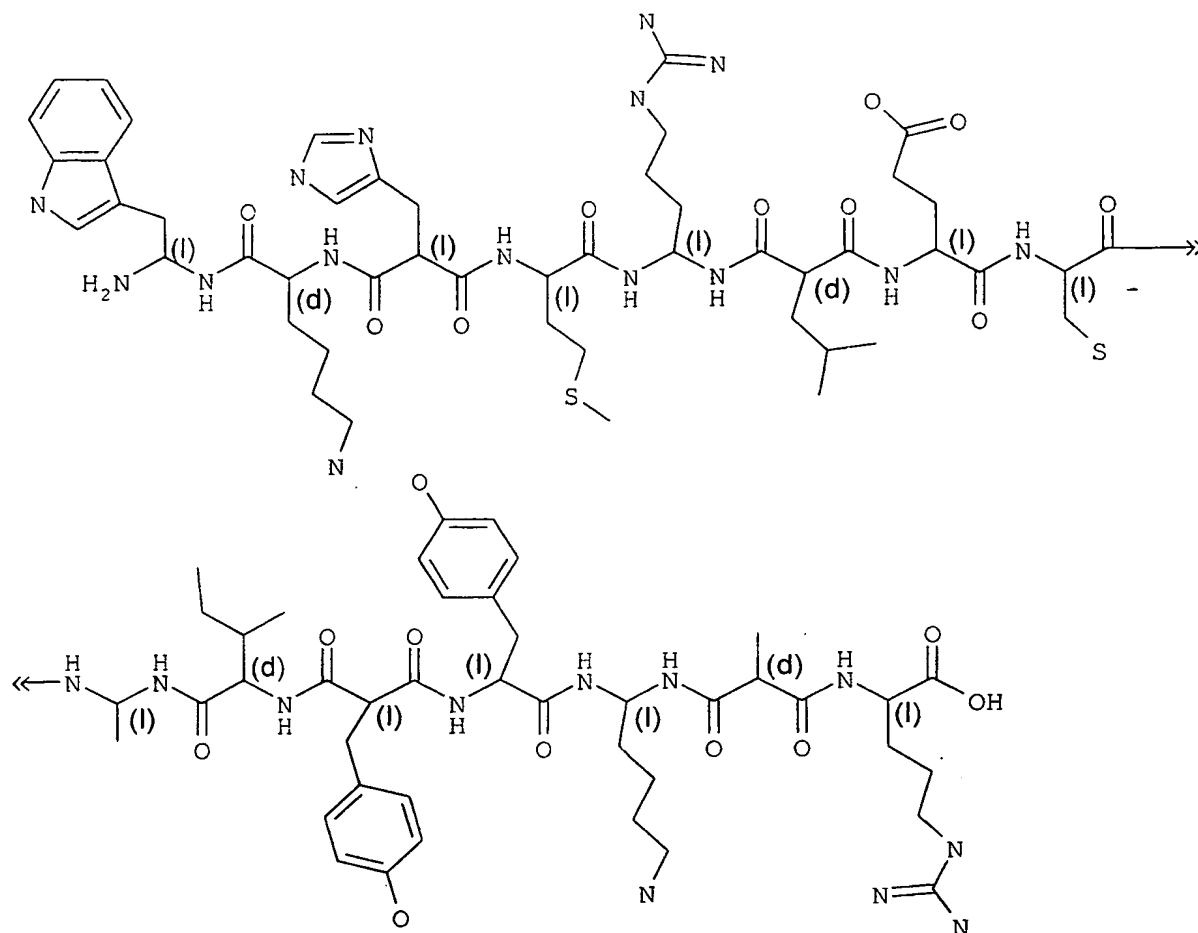


Furthermore, the methods of combinatorial chemistry are being brought to bear, e.g., by G.L. Verdine at Harvard University, on the development of new peptidomimetics. For example, one embodiment of a so-called "peptide morphing" strategy focuses on the random generation of a library of peptide analogs that comprise a wide range of peptide bond substitutes.

10



In an exemplary embodiment, the peptidomimetic can be derived as a retro-inverso analog of the peptide. To illustrate, the WKHMRLECAIYYKAR peptide (from HPV16) can be generated as the retro-inverso analog:



Such retro-inverso analogs can be made according to the methods known in the art, such as that described by the Sisto et al. U.S. Patent 4,522,752. For example, the illustrated retro-inverso analog can be generated as follows. The geminal diamine

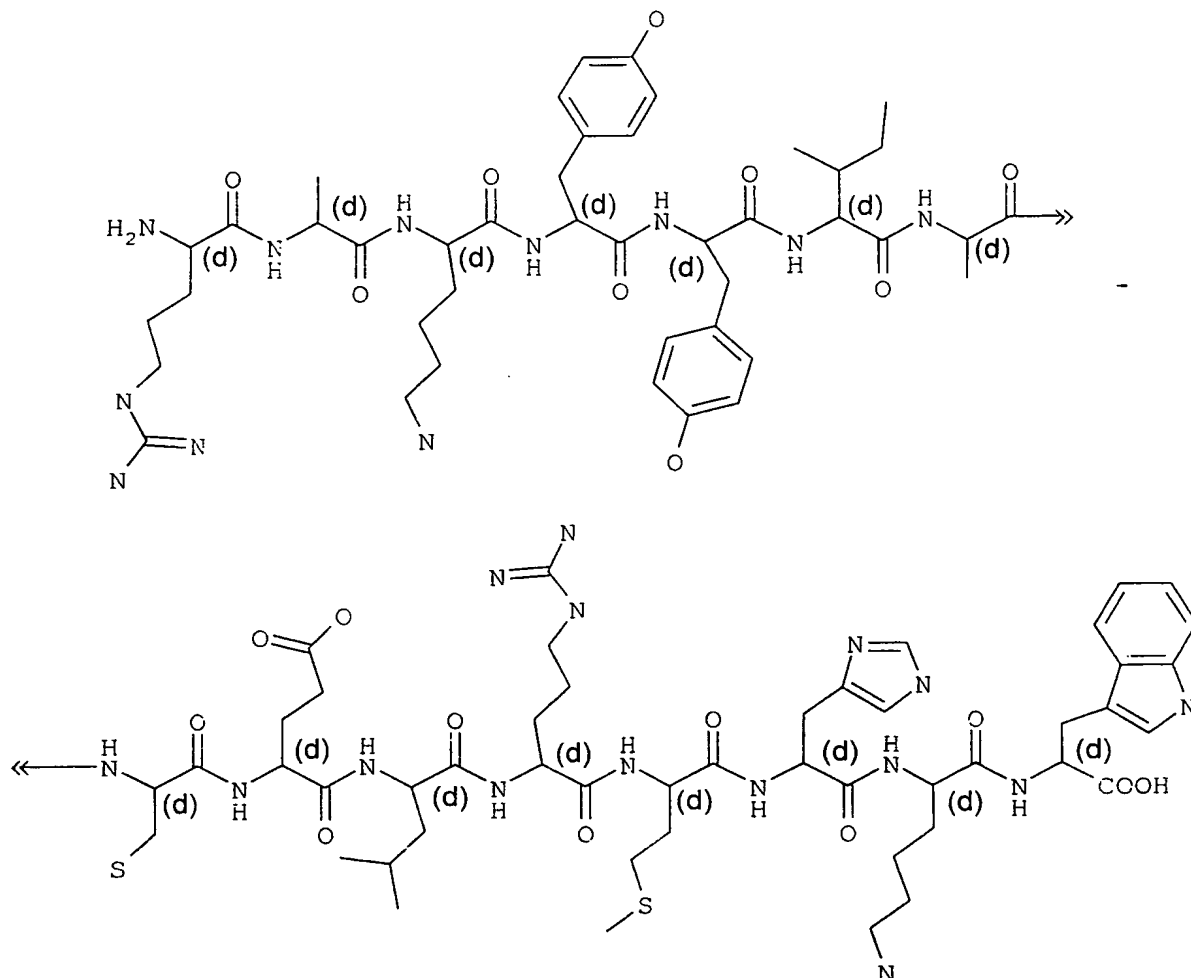
5 corresponding to the N-terminal tryptophan is synthesized by treating a protected tryptophan analog with ammonia under HOBT-DCC coupling conditions to yield the N-Boc amide, and then effecting a Hofmann-type rearrangement with I,I-bis-(trifluoroacetoxy)iodobenzene (TIB), as described in Radhakrishna et al. (1979) *J. Org. Chem.* 44:1746. The product amine salt is then coupled to a side-chain protected

10 (e.g., as the benzyl ester) N-Fmoc D-lys residue under standard conditions to yield the pseudodipeptide. The Fmoc (fluorenylmethoxycarbonyl) group is removed with piperidine in dimethylformamide, and the resulting amine is trimethylsilylated with bistrimethylsilylacetamide (BSA) before condensation with suitably alkylated, side-chain protected derivative of Meldrum's acid, as described in U.S. Patent 5,061,811 to

Pinori et al., to yield the retro-inverso tripeptide analog WKH. The pseudotripeptide is then coupled with with an L-methionine analog under standard conditions to give the protected tetrapeptide analog. The protecting groups are removed to release the product, and the steps repeated to enlogate the tetrapeptide to the full length
5 peptidomimetic. It will be understood that a mixed peptide, e.g. including some normal peptide linkages, will be generated. As a general guide, sites which are most susceptible to proteolysis are typically altered, with less susceptible amide linkages being optional for mimetic switching. The final product, or intermediates thereof, can be purified by HPLC.

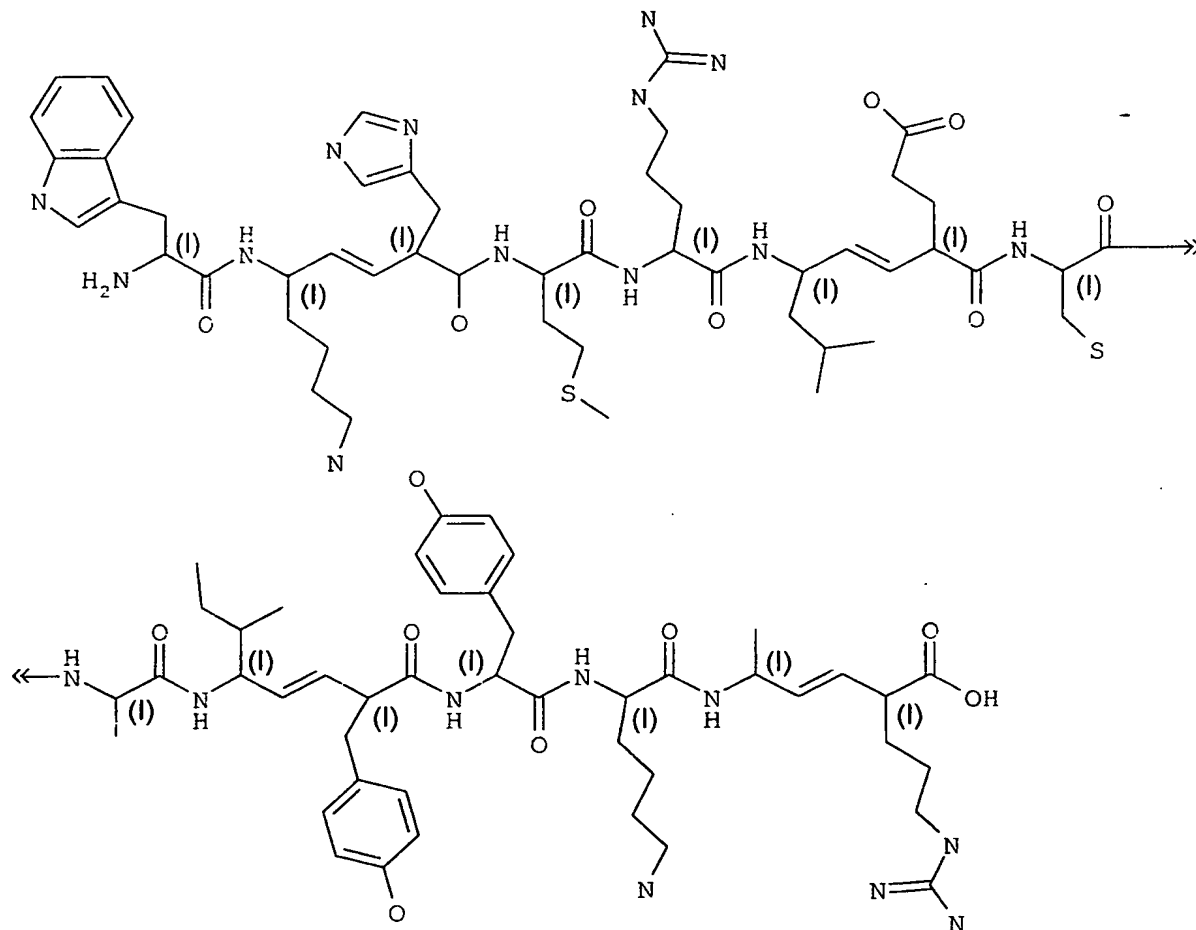
10 In another illustrative embodiment, the peptidomimetic can be derived as a retro-enatio analog of the peptide, such as the exemplary retro-enatio peptide analog derived for the illustrative WKHMRLECAIYYKAR peptide:

NH₂-(d)Arg-(d)Ala-(d)Lys-(d)Tyr-(d)Tyr-(d)Ile-(d)Ala-(d)Cys-(d)Glu-(d)Leu-(d)Arg-(d)Met-(d)His-(d)Lys-(d)Trp-COOH



Retro-enantio analogs such as this can be synthesized commercially available D-amino acids (or analogs thereof) and standard solid- or solution-phase peptide-synthesis techniques. For example, in a preferred solid-phase synthesis method, a suitably amino-protected (t-butyloxycarbonyl, Boc) D-trp residue (or analog thereof) is covalently bound to a solid support such as chloromethyl resin. The resin is washed with dichloromethane (DCM), and the BOC protecting group removed by treatment with TFA in DCM. The resin is washed and neutralized, and the next Boc-protected D-amino acid (D-lys) is introduced by coupling with diisopropylcarbodiimide. The resin is again washed, and the cycle repeated for each of the remaining amino acids in turn (D-his, D-met, etc). When synthesis of the protected retro-enantio peptide is complete, the protecting groups are removed and the peptide cleaved from the solid support by treatment with hydrofluoric acid/anisole/dimethyl sulfide/thioanisole. The final product is purified by HPLC to yield the pure retro-enantio analog.

In still another illustrative embodiment, trans-olefin derivatives can be made for the subject polypeptide. For example, an exemplary olefin analog is derived for the illustrative WKHMRLECAIYYKAR peptide:



- 5 The trans olefin analog of an E2 peptide can be synthesized according to the method of Y.K. Shue et al. (1987) *Tetrahedron Letters* 28:3225. Referring to the illustrated example, Boc-amino L-Ile is converted to the corresponding α -amino aldehyde, which is treated with a vinylcuprate to yield a diastereomeric mixture of alcohols, which are carried on together. The allylic alcohol is acetylated with acetic anhydride in
- 10 pyridine, and the olefin is cleaved with osmium tetroxide/sodium periodate to yield the aldehyde, which is condensed with the Wittig reagent derived from a protected tyrosine precursor, to yield the allylic acetate. The allylic acetate is selectively hydrolyzed with sodium carbonate in methanol, and the allylic alcohol is treated with triphenylphosphine and carbon tetrabromide to yield the allylic bromide. This

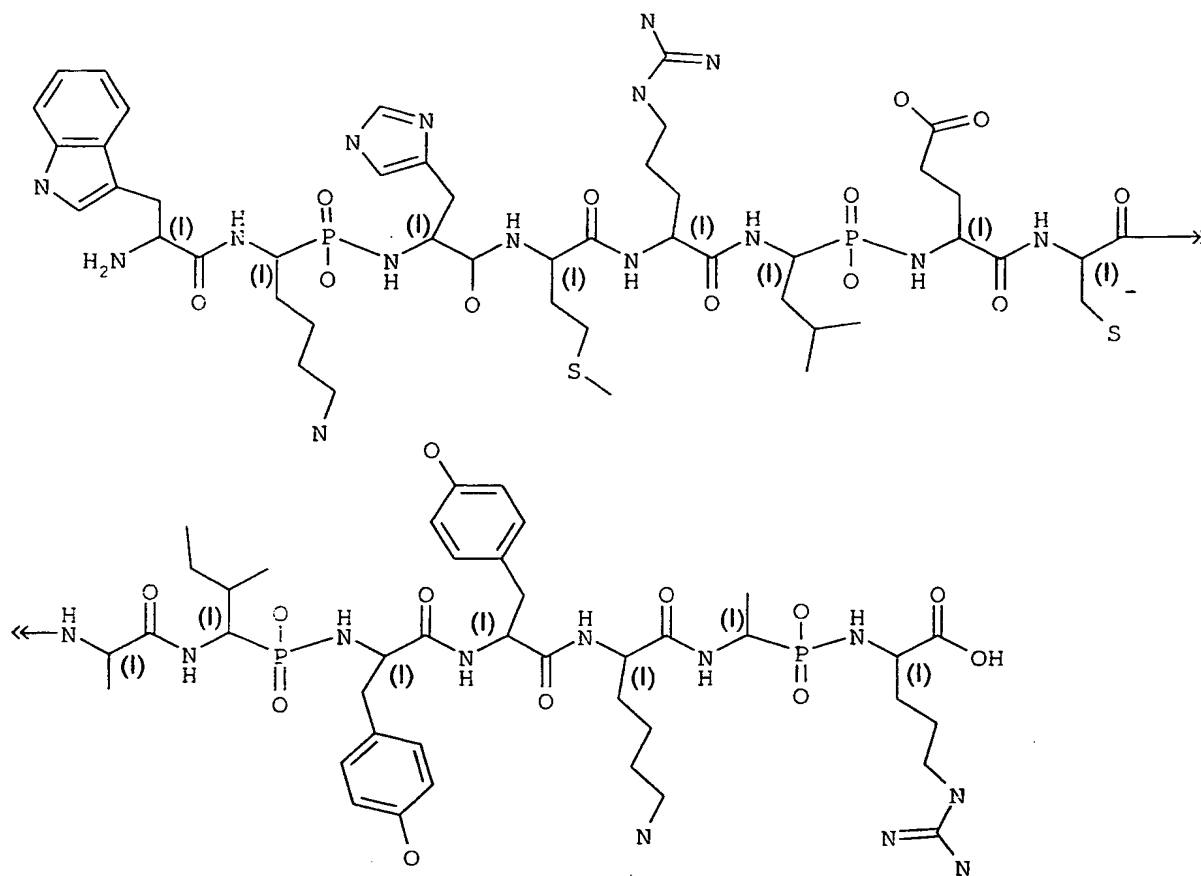
compound is reduced with zinc in acetic acid to give the transposed trans olefin as a mixture of diastereomers at the newly-formed center. The diastereomers are separated and the pseudodipeptide is obtained by selective transfer hydrogenolysis to unveil the free carboxylic acid.

5 The pseudodipeptide is then coupled at the C-terminus, according to the above example, with a suitably protected tyrosine residue, and at the N-terminus with a protected alanine residue, by standard techniques, to yield the protected tetrapeptide isostere A-I-Y-Y. The tetrapeptide is then further condensed with the olefinic tripeptide analog derived by similar means for Lys-Ala-Arg, and so forth to build up
10 the full E2 peptide. The protecting groups are then removed with strong acid to yield the desired peptide analog, which can be further purified by HPLC.

Other pseudodipeptides can be made by the method set forth above merely by substitution of the appropriate starting Boc amino acid and Wittig reagent. Variations in the procedure may be necessary according to the nature of the reagents used, but
15 any such variations will be purely routine and will be obvious to one of skill in the art.

It is further possible couple the pseudodipeptides synthesized by the above method to other pseudodipeptides, to make peptide analogs with several olefinic functionalities in place of amide functionalities. For example, pseudodipeptides corresponding to Met-Arg or Tyr-Lys, etc. could be made and then coupled together
20 by standard techniques to yield an analog of the E2 peptide which has alternating olefinic bonds between residues.

Still another class of peptidomimetic derivatives include the phosphonate derivatives, such as the partially phosphonate derivatized WKHMRLECAIYYKAR peptide:



The synthesis of such phosphonate derivatives can be adapted from known synthesis schemes. See, for example, Loots et al. in *Peptides: Chemistry and Biology*, (Escom Science Publishers, Leiden, 1988, p. 118); Petrillo et al. in *Peptides: Structure and*
 5 *Function* (Proceedings of the 9th American Peptide Symposium, Pierce Chemical Co. Rockland, IL, 1985).

Many other peptidomimetic structures are known in the art and can be readily adapted for use in the the subject E2 peptidomimetics. To illustrate, the E2 peptidomimetic may incorporate the 1-azabicyclo[4.3.0]nonane surrogate (see Kim et al. (1997) *J. Org. Chem.* 62:2847), or an *N*-acyl piperazic acid (see Xi et al. (1998) *J. Am. Chem. Soc.* 120:80), or a 2-substituted piperazine moiety as a constrained amino acid analogue (see Williams et al. (1996) *J. Med. Chem.* 39:1345-1348). In still other embodiments, certain amino acid residues can be replaced with aryl and bi-aryl moieties, e.g., monocyclic or bicyclic aromatic or heteroaromatic nucleus, or a
 15 biaromatic, aromatic-heteroaromatic, or biheteroaromatic nucleus.

The subject E2 peptidomimetics can be optimized by, e.g., combinatorial synthesis techniques combined with such high throughput screening as described herein.

Moreover, other examples of mimetopes include, but are not limited to, protein-based compounds, carbohydrate-based compounds, lipid-based compounds, nucleic acid-based compounds, natural organic compounds, synthetically derived organic compounds, anti-idiotypic antibodies and/or catalytic antibodies, or fragments thereof. A mimetope can be obtained by, for example, screening libraries of natural and synthetic compounds for compounds capable of inhibiting E1-E2 interaction. A mimetope can also be obtained, for example, from libraries of natural and synthetic compounds, in particular, chemical or combinatorial libraries (i.e., libraries of compounds that differ in sequence or size but that have the same building blocks). A mimetope can also be obtained by, for example, rational drug design. In a rational drug design procedure, the three-dimensional structure of a compound of the present invention can be analyzed by, for example, nuclear magnetic resonance (NMR) or x-ray crystallography. The three-dimensional structure can then be used to predict structures of potential mimetopes by, for example, computer modelling. the predicted mimetope structures can then be produced by, for example, chemical synthesis, recombinant DNA technology, or by isolating a mimetope from a natural source (e.g., plants, animals, bacteria and fungi).

VI. Pharmaceutical Preparations of E2 peptides and peptidomimetics

According to another aspect of this invention, E2 peptides and peptidomimetics may be administered directly to PV infected cells. Direct delivery of such E2 therapeutics may be facilitated by formulation of the peptidyl compound in any pharmaceutically acceptable dosage form, e.g., for delivery orally, intratumorally, peritumorally, interlesionally, intravenously, intramuscularly, subcutaneously, periolesionally, or (preferably) topical routes, to exert local therapeutic effects.

Topical administration of the therapeutic is advantageous since it allows localized concentration at the site of administration with minimal systemic adsorption.

This simplifies the delivery strategy of the agent to the disease site and reduces the extent of toxicological characterization. Furthermore, the amount of material to be applied is far less than that required for other administration routes. Effective delivery requires the agent to diffuse into the affected cells. Successful intracellular delivery of agents not naturally taken up by cells has been achieved by exploiting the natural process of intracellular membrane fusion, or by direct access of the cell's natural transport mechanisms which include endocytosis and pinocytosis (Duzgunes (1985) *Subcellular Biochemistry* 11:195-286). Such processes are also useful in the direct delivery and uptake of the subject E2 peptides and peptidomimetic by papillomavirus-infected cells.

In one embodiment, the membrane barrier can be overcome by associating the E2 protein in complexes with lipid formulations closely resembling the lipid composition of natural cell membranes. In particular, the subject E2 peptidomimetics are encapsulated in liposomes to form pharmaceutical preparations suitable for administration to living cells and, in particular, suitable for topical administration to human skin. The Yarosh U.S. Patent 5,190,762 demonstrates that proteins can be delivered across the outer skin layer and into living cells, without receptor binding, by liposome encapsulation.

These lipids are able to fuse with the cell membranes on contact, and in the process, the associated E2 peptidomimetic is delivered intracellularly. Lipid complexes can not only facilitate intracellular transfers by fusing with cell membranes but also by overcoming charge repulsions between the cell membrane and the molecule to be inserted. The lipids of the formulations comprise an amphipathic lipid, such as the phospholipids of cell membranes, and form hollow lipid vesicles, or liposomes, in aqueous systems. This property can be used to entrap the E2 peptidomimetic within the liposomes.

Liposomes offer several advantages: They are non-toxic and biodegradable in composition; they display long circulation half-lives; and recognition molecules can be readily attached to their surface for targeting to tissues. Finally, cost effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or

lyophilized product. has demonstrated the viability of this technology as an acceptable drug delivery system.

Liposomes have been described in the art as *in vivo* delivery vehicles. The structure of various types of lipid aggregates varies, depending on composition and method of forming the aggregate. Such aggregates include liposomes, unilamellar
5 vesicles, multilamellar vesicles, micelles and the like, having particle sizes in the nanometer to micrometer range. Methods of making lipid aggregates are by now well-known in the art. For example, the liposomes may be made from natural and synthetic phospholipids, glycolipids, and other lipids and lipid congeners: cholesterol,
10 cholesterol derivatives and other cholesterol congeners; charged species which impart a net charge to the membrane; reactive species which can react after liposome formation to link additional molecules to the liposome membrane; and other lipid soluble compounds which have chemical or biological activity.

In one embodiment, pH sensitive liposomes are a preferred type of liposome
15 for use with the present invention. One pathway for the entry of liposomes into cellular cytoplasm is by endocytosis into lysosomes of low pH. Accordingly, liposomes which are stable at neutral pH but release their contents at acidic pH can be used to deliver enzymes into the lysosomes of the cytoplasm, whereupon the contents are released.

20 Liposomes can be made sensitive to the low pH of the lysosomes by the lipid composition. In particular, pH sensitive liposomes can be prepared by using phospholipids which form lipid bilayers when charged but fail to stack in an ordered fashion when neutralized. An example of such a phospholipid is phosphatidylethanolamine, which is negatively charged above pH 9. The net charge of
25 a phospholipid can be maintained at a pH which would otherwise neutralize the head groups by including charged molecules in the lipid bilayer which themselves can become neutralized. Examples of these charged molecules are oleic acid and cholesteryl hemisuccinate, which are negatively charged at neutral pH but become neutralized at pH 5. The effect of combining these together in a lipid bilayer is that at
30 pH 9 all molecules are charged; at pH 7 the net negative charge of the oleic acid and cholesteryl hemisuccinate maintains the stability of the phosphatidylethanolamine,

and at pH 5 all components are protonated and the lipid membrane is destabilized. Additional neutral molecules, such as phosphatidylcholine, can be added to the liposomes as long as they do not interfere with stabilization of the pH sensitive phospholipid by the charged molecules.

5 In another embodiment, the E2 peptidomimetic is formulated with a positively charged synthetic (cationic) lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), in the form of liposomes, or small vesicles, which can fuse with the negatively charged lipids of the cell membranes of mammalian cells, resulting in uptake of the contents of the liposome (see, for
10 example, Felgner et al. (1987) *PNAS* 84:7413-7417; and U.S. Pat. No. 4,897,355 to Eppstein, D. et al.). Another cationic lipid which can be used to generate E2 peptidomimetic containing liposomes is the DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethyl-ammonio)propane (DOTAP) in combination with a phospholipid to form delivery vesicles.

15 Lipofectin™ (Bethesda Research Laboratories, Gaithersburg, Md.) and/or LipofectAMINETM, commercially available reagents, can be used to deliver the E2 peptidomimetic directly into cells. Positively charged complexes prepared in this way spontaneously attach to negatively charged cell surfaces, fuse with the plasma membrane, and can efficiently deliver functional E2 peptidomimetic into, for
20 example, keratinocytes. Sells et al. (1995) *Biotechniques* 19:72-76 describe a procedure for delivery of purified proteins into a variety of cells using such ploycationic lipid preparations.

 A significant body of information is emerging regarding the use of other cationic lipids for the delivery of macromolecules into cells. Other suitable lipid
25 vesicles for direct delivery of the E2 peptidomimetic include vesicles containing a quaternary ammonium surfactant (Ballas et al. (1988) *Biochim. Biophys Acta* 939:8-18); lipophilic derivatives of spermine (Behr et al. (1989) *PNAS* 86:6982-6986).

 The lipid formulations of the subject E2 peptidomimetic can be used in pharmaceutical formulations to deliver the E2 peptidomimetic by various routes and
30 to various sites in the animal body to achieve the desired therapeutic effect. Local or systemic delivery of the therapeutic agent can be achieved by administration

comprising application or insertion of the formulation into body cavities, inhalation or insufflation of an aerosol, or by parenteral introduction, comprising intramuscular, intravenous, intradermal, peritoneal, subcutaneous and topical administration.

Topical formulations are those advantageously applied to the skin or mucosa. Target mucosa can be that of the vaginal, cervical, vulvar, penal or anorectal mucosa, or target mucosa can be that of the gastrointestinal tract, comprising the mouth, larynx, esophagus and stomach. Other target tissues can be the accessible epidermal tissues which are infected by HPV. Lipids present in topical formulations can act to facilitate introduction of therapeutic E2 peptidomimetic into the target tissue, such as the stratum or corneum of the skin, by perturbing the barrier properties of the protective membrane, or by introducing perturbing agents or penetration enhancers such as DMSO, Azone™ or by promoting the activity of these penetration enhancers.

Other pharmaceutical formulations comprising the cationic lipids of the invention are topical preparations containing an anesthetic or cytostatic agent, immunomodulators, bioactive peptides or oligonucleotides, sunscreens or cosmetics. Preparations for topical use are conveniently prepared with hydrophilic and hydrophobic bases in the form of creams, lotions, ointments or gels; alternatively, the preparation may be in the form of a liquid that is sprayed on the skin. The effect of the cationic lipids is to facilitate the penetration of the active antiviral agent through the stratum corneum of the dermis.

The composition and form of pharmaceutical preparations comprising the liposome, in combination with the E2 peptidomimetic, can vary according to the intended route of administration.

Also, by suitable modifications of the liposome membranes, the liposomes can be made to bind to specific sub-populations of cells.

In still another embodiment, the therapeutic E2 peptidomimetic can be delivered by way of an artificial viral envelope (AVE). The art as described a number of viral envelopes which exploit molecular recognition of cell surface receptors by viral surface proteins as a means for selective intracellular delivery of macromolecules, including proteins. According to the method of Schreier, et. al., U.S.

Patent 5,252,348, a virtually unlimited number of artificial viral envelopes can be prepared and applied using recombinant or isolated surface determinants. For example, the AVEs be generated as viral mimetics of a number of human viruses including arboviruses; flaviviridae; bunyaviridae; hepatitis viruses; Epstein-Barr
5 viruses; herpes viruses; paramyxoviruses; respiratory syncytical virus; retroviruses including human T-lymphotrophic virus type I and II (HTLV-I/II) and human immunodeficiency virus type 1 and 2 (HIV- 1/2); rhinoviruses; orthopoxviruses; and human papilloma viruses (particularly those engineered not to express E6 and/or E7).

In another embodiment, direct delivery of a therapeutic E2 peptidomimetic
10 may be facilitated by chemical modification of the polypeptide itself. One such modification involves increasing the lipophilicity of the E2 peptidomimetic in order to increase binding to the cell surface, in turn, stimulating non-specific endocytosis of the protein. Lipophilicity may be increased by adding a lipophilic moiety (e.g., one or more fatty acid molecules) to the E2 peptidomimetic. A wide variety of fatty acids
15 may be employed. For example, the protein may be palmitoylated. Alternatively, a lipopeptide may be produced by fusion or cross-linking, to permit the E2 peptidomimetic to resemble the natural lipopeptide from E.coli, tripalmitoyl-S-glycerylcysteinyl-serine, at its amino terminus. This lipopeptide has been shown to increase the uptake of fused peptides (P. Hoffmann et al., (1988) *Immunobiol.*
20 177:158-70). Lipophilicity may also be increased by esterification of the protein at tyrosine residues or other amino acid residues. And uptake of the E2 peptidomimetic may be increased by addition of a basic polymer such as polyarginine or polylysine (Shen et al. (1978) *PNAS* 75:1872-76).

Direct delivery of E2 peptidomimetics according to this invention may also be
25 effected by the use of transport moieties, such as protein carriers known to cross cell membranes. For example, an E2 peptide may be fused to a carrier protein, preferably by a genetic fusion which may be expressed in a system such as E.coli, barulovirus or yeast. According to one embodiment of this invention, the amino terminus of the E2 peptide may be fused to the carboxy terminus of a transport moiety using standard
30 techniques.

Nucleotide sequences encoding such carrier-E2 peptide fusion proteins, operatively linked to regulatory sequences, may be constructed and introduced into appropriate expression systems using conventional recombinant DNA procedures. The resulting fusion protein may then be purified and tested for its capacity to (1) enter intact eukaryotic cells and (2) inhibit viral DNA replication once inside the intact eukaryotic cells.

In choosing a useful carrier protein, those of skill in the art will recognize the desirability of appropriate control experiments designed, *inter alia*, to test the possibility that the carrier portion of the fusion protein itself interacts with elements of the E1/E2 DNA replication transcriptional regulation system. If the carrier portion of the fusion protein is found to have undesirable interactions, such as enhancement of papillomavirus DNA replication, the portions of the carrier sequence responsible for these interactions should be identified and deleted in a way which permits the sequence to retain its carrier capacity. Alternately, one of several conventional carrier sequences which do not interact with elements of the E1/E2 DNA replications system can be substituted.

Useful carrier proteins include, for example, bacterial hemolysins or "blending agents", such as alamethicin or sulfhydryl activated lysins. Other carrier moieties which may be used include cell entry components of bacterial toxins, such as *Pseudomonas* exotoxin, tetanus toxin, ricin toxin, and diphtheria toxin. Also useful is melittin, from bee venom. Other useful carrier proteins include proteins which are viral receptors, cell receptors or cell ligands for specific receptors that are internalized, i.e., those which cross mammalian cell membranes via specific interaction with cell surface receptors, recognized and taken into the cell by cell surface receptors. Such cell ligands include, for example, epidermal growth factor, fibroblast growth factor, transferrin and platelet-derived growth factor. Alternatively, the ligand may be a non-peptide, such as mannose-6-phosphate, which permits internalization by the mannose-6-phosphate receptor. The transport moiety may also be selected from bacterial immunogens, parasitic immunogens, viral immunogens, immunoglobulins or fragments thereof that bind to target molecules, cytokines, growth factors, colony

stimulating factors and hormones. A transport moiety may also be derived from the tat protein of HIV-1.

As an alternative or addition to the above-described chemical modifications and protein carriers, which may be employed alone or in combination, other agents which allow penetration of the keratinized cell layer may be employed to facilitate delivery of the E2 peptidomimetics of this invention to papillomavirus-infected cells. In topical applications, for example, the E2 peptidomimetic may be administered in combination with dimethylsulfoxide, an agent which promotes penetration of cell membranes by substances mixed with it. Useful keratinolytic agents include, for example, salicylic acid, urea, and alpha -hydroxyacids. For such applications, the E2 peptidomimetic and any other agent may be administered topically, in cream or gel form.

According to an alternate embodiment of this invention, the therapeutic E2 peptidomimetic may be administered serially or in combination with other therapeutics used in the treatment of papillomavirus infections or diseases caused by them. Such therapeutics include interferons, such as IFN- γ , IFN- α and IFN- β derived from natural sources or produced by recombinant techniques, other cell mediators formed by leukocytes or produced by recombinant techniques such as for example, interleukin-1, interleukin-2, tumor necrosis factor, macrophage colony stimulating factor, macrophage migration inhibitory factor, macrophage activation factor, lymphotoxin and fibroblast growth factor. Alternatively, the E2 peptidomimetic may be administered serially or in combination with conventional therapeutic agents or regimens such as, for example, salicylic acid, podophyllotoxin, retinoic acid, surgery, laser therapy and cryotherapy. Such combination therapies may advantageously utilize less than conventional dosages of those agents, or involve less radical regimens, thus avoiding any potential toxicity or risks associated with those therapies.

It will also be understood by those skilled in the art that any of the above enumerated delivery methods may be augmented, where topical application is being carried out, by the use of ultrasound or iontophoretic delivery devices which facilitate transdermal delivery of proteins. See, for example, Banga et al. (1993) *Pharm Res* 10:697-702; and Mitragotri et al. (1995) *Science* 269:850-853.

VII. Gene Therapy

In another aspect, the present invention relates to gene therapy constructs containing a nucleic acid encoding a papillomavirus E2 peptide of the present invention, operably linked to at least one transcriptional regulatory sequence. The gene constructs of the present invention are formulated to be used as a part of a gene therapy protocol to deliver the subject therapeutic protein to a papillomavirus-infected or -transformed cell in an animal.

Any of the methods known to the art for the insertion of DNA fragments into a vector may be used to construct expression vectors consisting of appropriate transcriptional/ translational control signals and the desired E2 peptide-encoding nucleotide sequence. See, for example, Maniatis T., Fritsch E.F., and Sambrook J. (1989): *Molecular Cloning (A Laboratory Manual)*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; and Ausubel F.M., Brent R., Kingston R.E., Moore, D.D., Seidman J.G., Smith J.A., and Struhl K. (1992): *Current Protocols in Molecular Biology*, John Wiley & Sons, New York. These methods may include *in vitro* DNA recombinant and synthetic techniques and *in vivo* genetic recombination. Expression of a nucleic acid sequence encoding an E2 peptide may be regulated by a second nucleic acid sequence so that the peptide is expressed in a host infected or transfected with the recombinant DNA molecule. For example, expression of an E2 peptide may be controlled by any promoter/enhancer element known in the art. The promoter activation may be tissue specific or inducible by a metabolic product or administered substance.

Promoters/enhancers which may be used to control the expression of the E2 peptide *in vivo* include, but are not limited to, the native E2 promoter, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, *J. Exp. Med.*, 169:13), the human β -actin promoter (Gunning et al. (1987) *PNAS* 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al. (1984) *Mol. Cell Biol.* 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold

Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290:304-310; Templeton et al. (1984) *Mol. Cell Biol.* 4:817; and Sprague et al. (1983) *J. Virol.*, 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-797), the
5 herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) *PNAS* 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) *Nature Genetics*, 1:379-384), and Keratin gene promoters, such as Keratin 14.

Expression constructs of the subject E2 peptides may be administered in any biologically effective carrier, e.g. any formulation or composition capable of
10 effectively delivering the recombinant gene to cells *in vivo*. Approaches include insertion of the E2 peptide coding sequence in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin)
15 or derivatized (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or CaPO₄ precipitation carried out *in vivo*. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such
20 factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically.

A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid encoding the particular E2 peptide desired. Infection of cells with a viral vector has the advantage that a large proportion
25 of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., the recombinant E2 peptide, are expressed efficiently in cells which have taken up viral vector nucleic acid.

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a E2 peptide in the tissue of an
30 animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In

preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the E2 peptide-encoding gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

5 In clinical settings, the gene delivery systems for the therapeutic E2 peptide coding sequence can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from
10 specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see
15 U.S. Patent 5,328,470) or "gene gun" techniques. In preferred embodiments, the gene therapy construct of the present invention is applied topically to HPV infected or transformed cells of the skin or mucosal tissue. An E2 peptide gene construct can, in one embodiment, be delivered in a gene therapy construct by electroporation using techniques described, for example, by Dev et al. ((1994) *Cancer Treat Rev* 20:105-
20 115).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant
25 cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

Exemplification

The invention now being generally described, it will be more readily
30 understood by reference to the following examples which are included merely for

purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

In the results presented here, we have achieved replication of HPV16 DNA *in vitro*. This replication mimics that seen in transient *in vivo* replication assays, in that it requires interaction of E2 with E1 (31, 32). Furthermore, we demonstrate the ability of 5 23 or 15 amino acid peptides derived from the HPV16 E2 amino terminus to disrupt both interaction between E1 and E2, and papillomavirus DNA replication *in vitro*. The activity of such a peptide as a functional inhibitor in these assays has several implications: (i) it identifies a discrete region of E2 that is necessary and sufficient for specific interaction with E1; (ii) it provides proof-of-concept that a relatively small 10 molecule may be used to inhibit E1-E2 interaction and papillomavirus DNA replication; (iii) provides a starting point for the design or discovery of biologically active molecules capable of inhibiting papillomavirus DNA replication, of both low and high risk subtypes; (iv) provides a reagent that may facilitate functional dissection 15 of the dynamic processes that result in the stepwise assembly of the papillomavirus DNA replication initiation complex. Furthermore, we have utilized an alanine scanning approach to determine specific residues within the 15-amino acid peptide that are important to inhibition of E1-E2 interaction. For HPV-16E2 protein it was determined that residues R37, L38, E39 and I42 are important for the inhibitory 20 activity of the peptide. In addition, a fusion peptide in which the wild-type inhibitory peptide was fused to a membrane-translocating region of the *Drosophila* Antennapedia protein retained inhibitory activity comparable to the wild-type peptide. These observations may aid in the identification of effective bioactive anti-viral compounds that would inhibit HPV DNA replication.

25

Materials and Methods

Baculovirus expression of HPV E1 and E2 proteins. An NcoI-SmaI fragment from pUC-E116 (9) containing the HPV-16 E1 protein coding region was made blunt by using T4 polymerase and inserted into the blunt-ended BamHI site of 30 pBS-EE to generate pBS-EE-E116. [pBS-EE was generated by cloning the XbaI-BamHI fragment encoding the polyomavirus middle T antigen EE epitope (18) from pUC-

EE into pBluescript-SK (+) (Stratagene).] The Eco RI-NotI fragment from pBS-EE-E116 was recloned into the pVL1392 baculovirus transfer vector (Pharmingen). HPV-16 E2 and HPV-16 E2 E39A were cloned as Bam HI-Eco RI fragments into pVL 1392.

5 Recombinant baculovirus expressing HPV-11 EE-E1 and HPV-11 E2 were generously provided by Louise Chow. Hi-Five cells were infected at a multiplicity of 5 to 10 PFU per cell and incubated at 27°C for 48 h. Cells were scraped into ice-cold phosphate-buffered saline, pelleted by centrifugation in a Sorvall RT6000D tabletop centrifuge at 1,000 rpm for 5 min, washed once in ice-cold phosphate-buffered saline,
10 and repelleted. The cell pellet was then resuspended in hypotonic buffer (buffer A) containing 10 mM HEPES-K⁺ (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT) and protease inhibitor cocktail (PIC: 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10-µg/ml leupeptin, 10-µg/ml pepstatin A, 10-µg/ml phenanthroline, 16- µg/ml benzamidine). Cells were pelleted as before, resuspended
15 in buffer A containing 0.5 % Nonidet P-40 (NP-40), and incubated on ice for 10 min. Lysates were then spun at 4°C in a Sorvall tabletop microcentrifuge (3,000 rpm). The cytoplasmic extract supernatant was removed, and an equal volume of 50% (vol/vol) glycerol was added. Lysates were quick-frozen on dry ice and stored at - 70°C until use. The nuclear pellet was washed once in buffer A without NP-40, resuspended in
20 buffer C (20 mM HEPES-K⁺ [pH 7.9], 429 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% [vol/vol] glycerol, 0.5 mM DTT containing PIC), and incubated on ice for 30 to 40 min with intermittent vortexing. After clarification by centrifugation at full speed in a Sorvall microcentrifuge for 10 min at 4°C, the nuclear extract was quick frozen on dry ice and stored at - 70°C until use.

25 ***In vitro* HPV E1-E2 binding assay.** 300-500 ng protein from HPV16 or HPV11 EE-E1 expressing recombinant baculovirus infected cell nuclear extracts and 80-120ng from HPV E2 baculovirus infected cell nuclear extracts were incubated in 500µl NET-gel buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 0.1% NP-40, 1mM EDTA, 0.25% gelatin) at 4°C for one hour. 1-2µg anti-EE monoclonal antibody was
30 added and incubated with shaking at 4°C for one hour. Immune complexes were precipitated with protein-G agarose beads and washed twice for 30 minutes with ice

cold NET-gel buffer. Precipitated proteins were detected by Western blot using appropriate primary antibodies and anti-rabbit IgG HRP or anti-mouse IgG HRP (Amersham). Membranes were blocked for one hour in TNET (10mM Tris 7.5, 2.5 mM EDTA, 50mM NaCl, 0.1% Tween-20) containing 4%(w/v) dry milk. Washes and
5 antibody reactions were carried out in TNET, and proteins were visualized by chemiluminescence (Reneaissance ECL, NEN) and autoradiography. Bands were quantitated using the NIH image program.

Purification of HPV EE-E1 and E2 proteins. EE-E1 containing baculovirus infected cell nuclear extract (3-5mg total protein) containing 1% NP-40 was passed
10 over a mono-Q column (Econo-Pac Q cartridge, Bio-Rad, No.732-0025). The flowthrough was incubated at 4°C for one hour with 200µl anti-EE monoclonal antibody that had been covalently crosslinked to protein G sepharose beads. Beads were pelleted by centrifugation and washed once with 800µl (1%) NP-40 lysis buffer with proteinase inhibitor cocktail (PIC). Beads were then washed three times for ten
15 minutes at 4°C with 800µl high salt buffer (20mM Tris pH7.0, 0.5M NaCl, 0.5mM DTT, 0.1% NP-40, and PIC), followed by three ten minute washes with 800µl sodium phosphate buffer (10mM NaPO₄, pH 8.0, 0.5mM DTT, 0.1% NP-40, PIC). EE-E1 was eluted from the pelleted beads in 100mM triethylamine (pH 11.5). The beads were pelleted by centrifugation at 4°C, and the supernatant was dialyzed for 3 hours at 4°C
20 against one liter of dialysis buffer [HEPES-K+(pH 7.5), 1mM DTT, 10% glycerol, 150mM NaCl]. Dialysis buffer was then changed and dialysis was continued overnight at 4°C. This procedure yielded approximately 100-150µg/µl EE-E1 (>95% pure). 20µl aliquots of purified EE-E1 were quick-frozen on dry ice and stored at -70°C until use.

25 Baculovirus infected cell nuclear extract containing E2 in Buffer C (3mg/ml) was applied to an Econo-Pac Heparin column cartridge (Bio-Rad No. 732-0075) at 4°C. After washing with five column volumes of Buffer C containing proteinase inhibitor cocktail at a flow rate of 1ml/min. E2 was eluted in five column volumes of Buffer C containing 0.7M NaCl and 0.5ml column fractions were collected. Fractions
30 containing E2 were determined by Comassie staining of SDS-PAGE gels loaded with

aliquots of eluted fractions. This procedure yielded 300-500µg/µl E2, which was stored at -70°C until use.

***In vitro* replication reactions.** *In vitro* replication reactions were essentially as described by Kuo et al. (18) in 25µl reactions at 37°C for indicated times. Standard reactions were for 2 hours, and were terminated by addition of 200µl stop solution (20mM Tris-HCl pH 7.5, 10mM EDTA, 0.1% SDS, and 20µg/ml RNase A). After incubation at 37°C for 15 minutes, proteinase K was added (200µg/ml) and incubation was continued 30 minutes. Samples were extracted with 250µl phenol/chloroform/isoamyl alcohol (25:24:1), ethanol precipitated, washed with 70% ethanol, dried, resuspended in 20-30µl TE, and analyzed on a 0.8% agarose gel (TAE). The agarose gel was dried onto Hybond N+ membrane (Amersham), and replicated DNA was visualized by autoradiography. Bands were quantitated by Phosphorimager analysis. Replication templates were pKS7838-7905 (nt7838-7905/1-130) containing the HPV16 replication origin, and pUC7874-99 containing the HPV11 origin of replication (nt7874-7933/1-99). pUC7874-99 was a generously provided by Dr. Louise Chow.

Use of EE-EIC. The carboxy terminus of HPV16-E1, from amino acid 424 to 649, is the minimal domain necessary for interaction with E2 (Yasugi et al. 1997). An epitope tagged form of this domain (EE-EIC) was used in the E1-E2 interaction assays presented here. EE-EIC was shown to act the same as the full-length E1 protein with regard to inhibition by peptides (Kasukawa et al. 1998; unpublished data).

Fusion moieties (WP15 and membrane-translocating peptide). Fusion moieties were designed to contain the WP15 fused to either the membrane-translocating HIVderived Tat protein (aa 48-60) or the membrane-translocating *Drosophila* Antennapedia protein (aa 42-58). Synthesis of Tat-WP and -WP was carried out by Genosys Company (Texas). Both membrane-translocating carrier peptides were positioned at the aminoterminal and contained a Gly-Gly spacer between the transporter moiety and the WP 15 amino acid sequence.

***In vitro* HPV E1-E2 competition binding assay.** HPV-16 EE-EIC (240 ng) in 500 l NET gel buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% NP-40, 1

mM EDTA, 0.25% gelatin) were pre-incubated with 0 μ M, 10 μ M, 20 μ M, or 40 μ M peptide for 10 minutes on ice. The final concentration of DMSO, the peptide solvent, was kept constant in all samples. Nuclear extract containing HPV-16 E2 was then added, and the mixture was incubated with shaking at 4°C for 1 hour. Anti-EE
5 monoclonal antibody (1 mg/ml) (BAbCo) was added, and the mixture was incubated with shaking for 30 minutes. Immune complexes were precipitated with protein G-agarose beads for an additional 30 minutes at 4°C. The beads were washed with 500 l ice-cold NET-gel buffer three times.

10 ***In vitro* Replication of HPV16 DNA.**

Transient replication assays have demonstrated that interaction between HPV16 E1 and E2 is required for efficient origin dependent papillomavirus replication *in vivo* (31). E2 stimulation of BPV and HPV11 replication has been previously demonstrated *in vivo* (9, 19, 38, 40) and *in vitro* (18, 19, 43). We sought to
15 establish a system for the study of HPV16 DNA replication *in vitro*, and to determine whether specific interaction between HPV16 E1 and E2 was required for HPV16 DNA replication in this system. Figure 3A shows replication of an HPV16 ori containing plasmid *in vitro* using 293 cell extracts, along with partially purified HPV16 E2 and increasing amounts of affinity purified HPV16 EE-E1. As has been
20 documented for other papillomavirus E1 proteins, some replication was observed using relatively large amounts of E1 in the absence of E2 (lanes 2 through 7) (6, 16, 22, 33, 40, 48). Addition of 6ng of E2 greatly stimulated *in vitro* replication (lanes 8-13), whereas no replication was observed in the absence of EE-E1 (lane 1). Replication in this assay is ori-dependent, since the small amount of replication of the
25 pKS plasmid lacking the HPV16 origin detected at high concentrations of EE-E1 (Figure 3B, lanes 1-6) was not stimulated by E2 (Figure 1B, lanes 7-12). Quantitation of these results is shown in Figure 3C.

E1 Interaction is Required for E2 Stimulation of HPV16 Replication *In vitro*.

We have previously demonstrated that substitution of alanine for the conserved glutamic acid residue at amino acid residue 39 of HPV16 E2 (E39A) disrupts both HPV16 E1 interaction and transient HPV16 DNA replication in transient co-transfection assays. E39A is, however, fully competent as a transcriptional
5 activator (31). To test the capacity of this mutant E2 protein to support HPV16 DNA replication *in vitro*, increasing amounts of wild type HPV16 E2 or the E39A HPV16 E2 mutant protein were tested for *in vitro* replication activity in the presence of 20ng EE-E1 (Figure 4A). Wild type E2 greatly stimulated *in vitro* replication in a dose responsive manner, whereas the E39A mutant of HPV16 E2, which does not interact
10 with E1, had only a slight stimulatory effect, even at relatively high concentrations. This result demonstrated the dependence of E2 amino terminal mediated interaction with E1 in *in vitro* replication. Quantitation of these results is shown in Figure 4B.

Inhibition of Interaction Between HPV16 E1 and E2 *In vitro*.

15 The results described above suggested that a region of HPV16 E2 including the E39 residue might encompass a domain sufficient for interaction with E1. Therefore, we reasoned that a peptide encompassing this domain of HPV16 E2 might inhibit interaction between HPV16 E1 and E2, thereby inhibiting HPV16 DNA replication (Figure 5). In order to test this possibility, HPV16 E2 and epitope tagged
20 full length HPV16 E1 (EE16-E1) proteins were expressed separately using baculovirus vectors. EE-E1 and associated E2 proteins were immunoprecipitated using an anti-EE epitope monoclonal antibody. Precipitated E2 was detected by Western blot using polyclonal antiserum raised to the DNA binding domain of HPV16 E2 (35). As shown in lane 2 of Figure 6A, E2 co-immunoprecipitated with
25 EE16-E1, but was not precipitated by the EE epitope antibody in the absence of EE16-E1 (lane 1). Peptides encompassing HPV16 E2 amino acids 29-51 (E2N-WP23) or residues 33-47 (E2N-WP15) of HPV16 E2 were then tested for inhibition of E1-E2 interaction. These peptide sequences were derived from the sequence of HPV16 E2, and were based on an alignment of amino terminal residues that are
30 conserved among the papillomavirus E2 proteins (Figure 5). Lanes 3-9 of Figure 6A (upper panel) show the inhibitory effects of increasing concentrations of the E2N-

WP23 peptide. The smaller E2N-WP15 peptide displayed comparable inhibition of E1-E2 interaction (Figure 6A; lower panel, lanes 3-9). Both the HPV16 E2 protein itself and the E2 derived peptides act specifically in these assays, since the E39A replication defective HPV16 E2 mutant bound only slightly to EE16-E1 (lanes 14).
5 Furthermore, 23-mer and 15-mer peptides containing an amino acid substitution analogous to that of the HPV16 E2 E39A mutant did not inhibit E1-E2 interaction at concentrations at which the wild type peptide could do so (lanes 10-13). These peptides are named E2N-MP23 and E2N-MP15, respectively (see Figure 5). Quantitation of these results is shown in Figure 6B. The IC_{50} for inhibition of E1-E2
10 interaction by the E2N-WP23 and E2N-WP15 in these assays was 5mM and 7.5mM, respectively. In addition, when inhibition of E1-E2 interaction by peptide was plotted as a function of substrate concentration, this analysis indicated that the E2N-WP23 and E2N-WP15 peptides act through competitive inhibition (data not shown). We have previously shown that a domain spanning amino acids 421-647 of the HPV16 E1
15 carboxy terminus is necessary and sufficient for E2 interaction (45). Comparable and specific peptide inhibition of E2 interaction was observed using an epitope tagged form of this HPV16 E1 aa421-647 carboxyl terminal domain (data not shown).

Since the E2N-WP23 and E2N-WP15 peptides could inhibit interaction between HPV6 E1 and E2, their ability to inhibit *in vitro* HPV16 DNA replication
20 was also tested (Figures 7A and 7B). Figure 7C shows the compiled quantitative results of several independent experiments. Both the 23-mer and 15-mer forms of the wild type peptide could effectively inhibit *in vitro* replication. The inhibitory effects of E2N-MP23 and E2N-MP15 were also tested as specificity controls. Each of the wild type peptides inhibited replication much more effectively than the respective
25 E39A substituted analog, a result consistent with the importance of the E39 residue of HPV16 E2 in the interaction with E1. The replication inhibitory effects of the peptides are a result of inhibition of E1-E2 interaction, since the peptides had no effect on the low levels of replication observed with E1 alone (data not shown). Smaller peptides derived from sequences within the E2N-WP15 did not inhibit HPV16 E1-E2
30 interaction or replication *in vitro* (data not shown).

Peptide Inhibition of *In vitro* E1-E2 Interaction and Replication Is Not Limited to HPV16.

Very little is known about the physical properties of domains within the papillomavirus E1 and E2 proteins that are involved in intermolecular recognition and association. Within the sequence of the HPV16 E2 derived 15 amino acid minimal peptide that inhibits HPV16 E1-E2 interaction, there are seven well conserved residues (W-X-X-M/V/I-R-X-E-X-X-I/L-X-X-X-X-A-R) (see also Figure 5). We reasoned that if these residues adequately represented a conserved E1 interaction domain, the HPV16 E2 derived peptide might also inhibit interaction between other HPV E1 and E2 proteins. We therefore tested the inhibitory effects of the E2N-WP15 peptide using the HPV11 E1 and E2 proteins. Epitope tagged versions of the HPV11 (HPV11 EE-E1) and HPV16 E1 (HPV16 EE-E1) proteins were tested for binding to HPV11 and HPV16 E2. The effect of the E2N-WP15 peptide on these interactions was also assessed. As shown in Figure 7A, E2N-WP15 effectively inhibited interaction between HPV11 E1 and HPV16 E2 (lanes 10-13), along with HPV11 E1/E2 interaction (lanes 20-24). As was the case for interaction between HPV16 E2 and HPV16 E1, the HPV16 E2 E39A mutant did not bind HPV11 E1 (lanes 8 and 16). The E2N-MP15 peptide did not inhibit HPV 11/11 E1-E2 interaction or interaction between HPV11 E1 and HPV16 E2. This suggests that the domain of HPV16 E2 that interacts with HPV16 E1, its natural counterpart, can also interact specifically with HPV11 E1. Interaction between HPV11 E1 and HPV16 E2 was not observed (data not shown). This is consistent with the inability of HPV16 E1 and HPV11 E2 to support transient replication in co-transfection assays (47). Thus, although many elements of E1-E2 interaction are conserved among the HPV subtypes, these interactions are not completely interchangeable. Further examination of the nature of this non-reciprocal functionality may reveal important determinants in the specificity of E1-E2 interaction.

The ability of the E2N-WP15 peptide to inhibit HPV11 E1 mediated replication *in vitro* was also tested. As shown in Figure 7B (lanes 1-9), HPV11 E1 could cooperate with HPV16 E2 in supporting dose responsive *in vitro* replication of the HPV11 ori. (These proteins also functioned in replication of the HPV16 origin,

but less efficiently than the HPV11 ori: data not shown). The E2N-WP15 peptide inhibited HPV16 E2/HPV11 E1 dependent replication of the HPV11 origin with approximately the same effectiveness as its inhibition of HPV16 E1/E2 mediated *in vitro* replication (Figure 7B, lanes 11-16). The E39A HPV16 E2 mutant had only a slight effect on replication in these assays (lanes 17-19). Thus, the effects of inhibitory peptides on *in vitro* replication mirrors those observed in E1-E2 interaction assays.

The Dynamics of E1-E2 Interaction

Alanine substitutions. In order to discern the effect of each residue on the ability of the peptide to inhibit HPV-16 E1-E2 interaction, substitution with alanine at each position of the 15-amino acid inhibitory peptide was performed. Alanine scanning mutagenesis generates a small and systematic set of mutant peptides whose inhibitory activity can be readily tested using an *in vitro* competition binding assay (Cunningham et al., *Science*, 244:1081, 1989). Alanine substitution does not impose new structural effects related to hydrogen bonding, unusual hydrophobicity, or steric bulk, and it is expected to cause minimal perturbation of secondary structure; alanine is compatible with all secondary structures in both buried and solvent-exposed positions (Abroji et al., *J. Virology*, 70(9):6169, 1996; Cunningham et al., *Science*, 244: 1081, 1989; Rose et al., *Science*, 229:834, 1985; Klapper et al., *Biochem Biophys Res Commun.* 78(3):1018, 1977; Chothia et al., *J. Molecu Biol.* 105(1):1, 1976). Also, in contrast to amino acid deletions, substitution with alanine preserves the original spacing of residues. Thus, alanine scanning is a useful technique for isolating the effect of one amino acid within the context of an existing peptide or protein structure.

Little is known about the ordered assembly of the papillomavirus E1 and E2 proteins at the origin of replication, and the formation of the replication initiation complex. Two types of BPV E1-E2-ori complexes have been observed. One complex contains both BPV E1 and E2 (22, 34); the other complex contains only E1, which forms an oligomeric ring around the DNA (33). Although this latter complex can form at high E1 concentrations in the absence of E2 (22, 34), E2 stimulates assembly of the oligomeric E1 complex.

As a preliminary step in examining the assembly of replication factors in HPV16 DNA replication, we performed experiments to determine the timing of E1-E2 complex formation. As shown in Figure 9, HPV16 E1-E2 complexes form rapidly; the binding reaction was 50% complete after approximately 3 minutes, and this binding reached a saturated steady state within 10 minutes (lanes 2-7). We also sought to utilize the E2N-WP15 peptide to study the E1-E2 binding reaction itself by adding peptide (50 μ M final concentration) at various times after starting the E1-E2 binding reaction. Addition of peptide at 5 minutes resulted in significant inhibition of subsequent E1-E2 interaction, but addition of peptide at or after 10 minutes had only a minimal effect on the stability of this E1-E2 interaction. We interpret this to mean that complex formation between HPV16 E1 and E2 is rapid, and that the E2N-WP15 inhibits formation of this complex, but has no effect upon the E1-E2 complex in the binding reaction after its formation.

15 **Testing of Tat- and Antennapedia-WP15 fusion peptides for inhibition of E1-E2 interaction**

Rationale. Although the full-length HPV-16 E2 protein and its various mutant forms have been tested *in vivo* for both transcriptional activation and transient replication activity, analogous studies of the WP15 E2 peptide have not been attempted. The characterization of the efficacy and activity of WP15 *in vivo* is an important goal; however, efficient and reliable introduction of peptides into mammalian cells is technically difficult.

WP 15 fusion peptides that contained portions of either the HIV Tat or *Drosophila* Antennapedia protein (Ant) were designed and synthesized (Figure 13). Small peptides derived from both Tat and Ant have been shown to translocate through mammalian cell plasma membranes and localize in the nucleus. It has been demonstrated that peptides chemically coupled to Tat become functionally internalized into the nuclei of several mammalian cell lines (Vives et al., *JBC*, 272:16010-17, 1997). Similarly, a 16-amino acid polypeptide derived from the third helix of Antennapedia translocates into the nucleus in an energy-independent mechanism (Derossi et al., *J. Biol. Chem.*, 269:10444, 1994; Schurze-Redelmeier et

al., *J. of Immunology*, 157:650, 1996; Bonfanti et al., *Cancer Research*, 57:1442, 1997).

As a pre-requisite for testing the peptides for inhibition of papillomavirus DNA replication *in vivo*, fusion peptides were first tested for their ability to inhibit HPV-16 E1-E2 interaction *in vitro*.

Antennapedia fusion peptide retains the original inhibitory activity of WP15. A second membrane-translocating peptide, Antennapedia protein (Ant) was also linked to the wild-type peptide and tested in the *in vitro* inhibition of E1-E2 binding assay. The Ant-WP peptide inhibited HPV-16 E1-E2 interaction with levels comparable to that of wild-type peptide (Figure 14). Based upon the above results, it would be expected that E39A would also inhibit HPV replication *in vitro*.

Tat fusion peptide interferes with inhibitory activity of wild-type peptide. The first peptide tested was derived from the HIV-1 Tat basic protein domain. This fusion moiety was tested in the *in vitro* inhibition of E1-E2 binding assay to determine whether or not linkage to the translocating peptide affected the activity of the wild-type inhibitory peptide. E1-E2 complex formation was not inhibited in the presence of 10 μ M, 20 μ M, and 40 μ M of Tat-WP, suggesting that linkage to the Tat peptide, completely disrupted the inhibitory activity of the wild-type peptide (Figure 14).

20

Discussion

The papillomavirus E1 and E2 proteins carry out the critical step necessary for viral DNA replication: assembly of the replication initiation complex at the origin of replication. This process requires specific interaction of the E2 amino terminus with the E1 protein. Papillomavirus replication is otherwise dependent on host cell polymerases and other components of the host cell replication machinery.

We have mapped a number of viral DNA replication associated activities within the HPV16 E1 protein (45). The E2 interaction domain of HPV16 E1 resides within the carboxy-terminal 223 amino acids (aa421-647), a region that also includes the ATP binding domain. This region is also capable of interacting with other E1

30

molecules in two-hybrid assays (46). Many missense mutations within this domain of HPV16 E1 simultaneously disrupt multiple E1 activities, including transient replication, E1-E1 association, formation of E1-E2 complexes, ATPase activity, and the ability of E1 to interact with cellular proteins in two-hybrid assays. The
5 pleiotropic effects of many of these HPV16 E1 mutations have led us to speculate that such E1 functions are tightly clustered or functionally interdependent (46).

The initial deletion mapping studies of the BPV1 E2 amino terminus identified domains necessary for transcriptional activation and viral DNA replication (24, 42), but failed to discriminate between these two E2 functions. Non-overlapping deletions
10 within the conserved amino terminus of BPV E2 (e.g. aa1-15, aa92-161, and aa195-282) disrupted both transcriptional activation and replication functions. These results implied either that the E2 transcriptional activation and replication functions co-exist within an extended domain of the amino terminus, or that such deletion mutations caused concurrent disruption of multiple functional moieties within the amino
15 terminus.

More recent studies of the HPV16 E2 and BPV1 E2 amino terminal domains that employed alanine substitution mutagenesis successfully separated the E2 amino terminal replication and transcriptional activation functions. Mutation of the conserved glutamic acid residue at position 39 of HPV16 E2 to alanine specifically
20 abrogates its ability to stimulate transient papillomavirus replication. This mutant no longer interacts with E1, but is proficient for transcriptional activation. Alternatively, substitution of alanine for the conserved isoleucine at position 73 of HPV16 E2 abrogates transcriptional activation, but has no effect on E1 interaction and DNA replication (31). Similar directed mutagenesis studies have extended these results to
25 BPV E2 (1, 6, 7, 10). A genetic screen based on BPV E2 transcriptional activation in yeast has identified additional E2 mutants that have lost either or both of these functions (6, 13).

Such studies have provided convincing evidence that the papillomavirus E2 amino terminus is composed of at least two separable functional domains: one domain
30 defined by isoleucine 73 that is required for transcriptional activation, and a separate domain defined by the glutamic acid residue at position 39 that is required for E1

interaction and replication. In this sense, it is notable that the BPV E2 deletion mutants mentioned above that disrupted both transcriptional activation and replication overlap neither the E39 nor I73 domain. Indeed, even missense mutations scattered throughout the first 200 amino acids of E2 are capable of abrogating either or both functions (1, 6, 13, 31, 42). Thus, although the transcriptional activation and replication functions of the E2 amino terminus are functionally independent, the domains required for these respective functions do not necessarily retain their functional integrity when removed from their normal context. Furthermore, in our mapping studies of HPV16 E2, we defined a domain encompassing amino acids 1-90 as the smallest domain capable of interaction with HPV16 E1 in two hybrid assays (45). For these reasons, it was uncertain whether a peptide derived from the E39 region of HPV16 E2 would be capable of competing with intact E2 proteins for specific interaction with the HPV16 E1 carboxy terminus. In this study, we demonstrate that a fifteen amino acid peptide derived from the amino terminus of HPV16 E2 and centered upon E39 can inhibit both HPV16 E1-E2 interaction and papillomavirus replication *in vitro*. This inhibition is specific, since an equivalent peptide containing an alanine substitution at position 39 had minimal inhibitory activity in both assays. The ability of this peptide to act as a competitive inhibitor of *in vitro* E1-E2 interaction suggests that a specific E1 recognition activity is contained within a relatively small part of the E2 amino terminus and that this domain is able to adopt a conformation suitable for occupancy of a critical E2 binding site of E1.

The E1-E2 interaction and peptide inhibition experiments presented here indicate functional conservation in E1-E2 recognition and interaction between the E1 and E2 proteins of HPV11 and HPV16. The E39 residue of E2 seems to be a conserved functional component of E1-E2 interaction among these proteins, since this residue is required for interaction between HPV16 E1 and E2, as well as HPV11 E1 and HPV16 E2. E39 is also critical for the replication function of BPV-1 E2 (10). Our peptide inhibition experiments have reiterated the importance of this residue in both HPV16 and HPV11 E1-E2 interaction and replication, since E2N-WP15 disrupted interaction between all combinations of HPV16 and HPV11 E1 and E2 proteins. The lack of interaction we observed between HPV11 E1 and HPV16 E2 in binding assays

is consistent with the inability of these proteins to support transient replication when transfected into 293 cells (47). HPV16 E1 seems to be particularly selective in selection of its E2 partner, as compared to HPV11 E1, which can cooperate functionally in replication assays with the E2 proteins of HPV6, HPV11, HPV16, or HPV18 (9, 38). Experiments using HPV11 and HPV16 chimeric E1 proteins have identified the carboxyl-terminal 284 amino acids of HPV16 E1 as the domain that mediates selective binding of HPV16 E1 to HPV16 E2 (47). This selectivity has been attributed to steric interference within this domain of HPV16 E1 that somehow prevents HPV11 E2 binding. Testing the ability of an extended set of peptides derived from the E39 region of a variety of E2 proteins to inhibit interaction between other E1 and E2 proteins may provide greater insight into the exact nature of this selectivity and thus allow an estimation of the spectrum of HPV subtypes against which an individual peptide or small molecule might be effective.

We also used the E2N-WP15 peptide to examine early events in formation of E1-E2 complexes and initiation of viral DNA replication. Formation of the HPV16 E1-E2 complex occurred quickly, within 10 minutes, under the conditions of the *in vitro* E1-E2 interaction assay.

Demonstration that a peptide could inhibit *in vitro* E1-E2 interaction and papillomavirus DNA replication constitutes an essential first step in assessing the potential efficacy of small compounds as papillomavirus antiviral agents and validates this approach to the design of papillomavirus antiviral compounds.

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All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more
10 than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We claim

1. A method of treating a cell infected with a papillomavirus (PV), comprising contacting the cell with a preparation comprising an effective amount of a small
5 organic compound which can competitively inhibit interaction of a PV E2 protein with a PV E1 protein.
2. A method of treating a mammal for a papillomavirus-induced condition, or preventing said condition, comprising administering to an mammal a
10 pharmaceutical preparation comprising a therapeutically effective amount of a small organic compound which can competitively inhibit interaction of a papillomavirus (PV) E2 protein with a PV E1 protein, which compound is formulated in the pharmaceutical preparation for delivery into papillomavirus-infected cells of the animal.
- 15 3. The method of claim 1 or 2, wherein the compound has a molecular weight of less than 10,000 amu.
4. The method of claim 1 or 2, wherein the compound has a molecular weight of
20 less than 5000 amu.
5. The method of claim 4, wherein the compound has a molecular weight of less than 3000 amu.
- 25 6. The method of claim 1 or 2, wherein the compound is a peptide or peptidomimetic.

7. The method of claim 1 or 2, wherein the compound is a peptide or peptidomimetic comprising 3-25 residues.
8. The method of claim 6, wherein the compound is peptidomimetic comprising, as
5 a surrogates for one or more amide bonds, a trans-olefin, a fluoroalkene, a methyleneamino, a phosphonamides, or a sulfonamide.
9. The method of claim 6, wherein the compound comprises a core sequence
10 having the formula W-X(1)-X(2)-X(3)-R-X(4)-E-X(5)-X(6)-X(7)-X(8)-X(9)-
X(10)-A-X(11).
wherein
- W represents a tryptophan residue, or an analog thereof;
- R represents an arginine residue, or an analog thereof;
- E represents a glutamic acid residue, or an analog thereof;
- 15 A represents an alanine residue, or an analog thereof
- X(1) represents an amino acid residue having a polar sidechain;
- X(2), X(5), X(6), X(7) and X(8) each, independently, represent an amino acid residue having a neutral sidechain;
- X(3) represents an amino acid residue having a hydrophobic sidechain;
- 20 X(4) and X(10) each, independently, represent an amino acid residue having a basic sidechain or a neutral sidechain;
- X(9) represents an amino acid residue having an aromatic sidechain; and
- X(11) represents an amino acid residue having a basic sidechain.
- 25 10. The method of claim 9, wherein
- X(1) represents arg, asn, asp, cys, glu, gln, his, lys, ser, thr or tyr, or an analog thereof;

X(2), X(5), X(6), X(7) and X(8) each, independently, represent ala, asn, cys, gln, gly, his, ile, leu, met, phe, pro, ser, thr, trp, tyr, or val, or an analog thereof;

X(3) represents ala, gly, ile, leu, met, phe, pro, trp, tyr, or an analog thereof;

5 X(4) and X(10) each, independently, represent arg, his, lys, ala, asn, cys, gln, gly, his, ile, leu, met, phe, pro, ser, thr, trp, tyr or val, or an analog thereof;

X(9) represents his, phe, trp or tyr, or an analog thereof; and

X(11) represents arg, his or lys, or an analog thereof.

10 11. The method of claim 9 or 10, wherein X(4) represents an amino acid residue having a neutral hydrophobic sidechain; X(5) represents an amino acid residue having a neutral polar sidechain; X(6) and X(7) each independently represent an amino acid residue having neutral aliphatic sidechain; and X(8) represents an amino acid residue having a large hydrophobic sidechain.

15 12. The method of claim 9 or 10, wherein: X(1) represents gln, lys, tyr, cyd or thr or an analog thereof; X(2) represents leu, thr, cys, tyr, his, ala or cys or an analog thereof; X(3) represents met, leu, ile, val, thr or arg or an analog thereof; X(4) represents leu, lys, his, tyr, trp, arg, val, ile, met, gln or thr or an analog thereof; X(5) represents gln, ala, ser, cys, asn or tyr or an analog thereof; X(6) represents ala, val or thr or an analog thereof; X(7) represents leu, ile or val or an analog thereof; X(8) represents leu, tyr, phe, met or gln or an analog thereof; X(9) represents tyr, his or phe or an analog thereof; X(10) represents lys, tyr, thr or ala or an analog thereof; and
20 X(11) represents arg or lys or an analog thereof.

25 13. The method of claim 6, wherein the compound comprises a core sequence having the formula: R-X(4)-E-X(5)-X(6)-X(7), wherein:

R represents an arginine residue, or an analog thereof;

E represents a glutamic acid residue, or an analog thereof;

X(4) represents an amino acid residue having a basic sidechain or a neutral sidechain; and

X(5), X(6), and X(7) each independently represent an amino acid residues having a neutral side chain.

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14. The method of claim 13, wherein X(4) represents leu, trp, gln, his, met, or thr, or an analog thereof; X(5) represents cys, asn, gln or ser, or an analog thereof; X(6) represents ala, val, or thr, or an analog thereof; and X(7) represents ile, leu, val, or met, or an analog thereof.

10

15. The method of claim 1, wherein the cell is contacted with compound *in vitro*.

16. The method of claim 1, wherein the cell is contacted with compound *in vivo*.

15 17. The method of claim 2, wherein the mammal is a livestock, zoo animal or pet.

18. The method of claim 2, wherein the mammal is a human.

19. The method of claim 18, wherein the human is infected with a human papillomavirus (HPV).
20

20. The method of claim 19, wherein the HPV is a high risk HPV.

21. The method of claim 20, wherein the high risk HPV is selected from the group consisting of HPV-16, HPV-18, HPV-31 and HPV-33.
25

22. The method of claim 19, wherein the HPV is a low risk HPV.

23. The method of claim 2, wherein the papillomavirus-induced condition being treated includes growth of papillomavirus preneoplastic and neoplastic lesions.
- 5
24. The method of claim 2, wherein the papilloma-induced condition being treated includes the growth of cutaneous lesions selected from the group consisting of warts and other benign cutaneous lesions.
- 10
25. The method of claim 2, wherein the papillomavirus-induced condition being treated is selected from the group consisting of Plantar warts (verruca plantaris), common warts (verruca plana), Butcher's common warts, flat warts, genital warts (condyloma acuminatum), and epidermodysplasia verruciformis.
- 15
26. The method of claim 2, wherein the papillomavirus-induced condition being treated is selected from the group consisting of laryngeal, oral, pharyngeal, esophageal and and other upper airway papilloma.
- 20
27. The method of claim 2, wherein the papillomavirus-induced condition being treated is selected from the group consisting of vaginal, cervical, vulvar, penile and anorectal carcinoma.
- 25
28. The method of claim 1 or 2, wherein the compound is formulated for delivery into keratinocytes.
29. The method of claim 2, wherein the compound is formulated for delivery into cutaneous or mucosal tissue.

30. The method of claim 1 or 2, wherein the compound is formulated in liposomes.
31. A pharmaceutical preparation comprising a small organic compound which can
5 competitively inhibit interaction of a PV E2 protein with a PV E1 protein.
32. The preparation of claim 31, wherein the compound has a molecular weight of less than 10,000 amu.
- 10 33. The preparation of claim 32, wherein the compound has a molecular weight of less than 5000 amu.
34. The preparation of claim 29, wherein the compound is a peptide or peptidomimetic.
- 15 35. The preparation of claim 34, wherein the compound is peptidomimetic comprising, as a surrogate for one or more amide bonds, a trans-olefin, a fluoroalkene, a methyleneamino, a phosphonamides, or a sulfonamide.
- 20 36. The preparation of claim 34, wherein the compound is a peptide or peptidomimetic comprising 3-25 residues.
37. The preparation of claim 34, wherein the W-X(1)-X(2)-X(3)-R-X(4)-E-X(5)-X(6)-X(7)-X(8)-X(9)-X(10)-A-X(11), compound comprises a core sequence
25 having the formula:
wherein

W represents a tryptophan residue, or an analog thereof;

R represents an arginine residue, or an analog thereof;

E represents a glutamic acid residue, or an analog thereof;

A represents an alanine residue, or an analog thereof

X(1) represents an amino acid residue having a polar sidechain:

5 X(2), X(5), X(6), X(7) and X(8) each, independently, represent an amino acid residue having a neutral sidechain;

X(3) represents an amino acid residue having a hydrophobic sidechain;

X(4) and X(10) each, independently, represent an amino acid residue having a basic sidechain or a neutral sidechain;

10 X(9) represents an amino acid residue having an aromatic sidechain; and

X(11) represents an amino acid residue having a basic sidechain.

38. The preparation of claim 34, wherein

15 X(1) represents arg, asn, asp, cys, glu, gln, his, lys, ser, thr or tyr, or an analog thereof;

X(2), X(5), X(6), X(7) and X(8) each, independently, represent ala, asn, cys, gln, gly, his, ile, leu, met, phe, pro, ser, thr, trp, tyr, or val, or an analog thereof;

X(3) represents ala, gly, ile, leu, met, phe, pro, trp, tyr, or an analog thereof;

20 X(4) and X(10) each, independently, represent arg, his, lys, ala, asn, cys, gln, gly, his, ile, leu, met, phe, pro, ser, thr, trp, tyr or val, or an analog thereof;

X(9) represents his, phe, trp or tyr, or an analog thereof; and

X(11) represents arg, his or lys, or an analog thereof.

25 39. The preparation of claim 34 or 35, wherein X(4) represents an amino acid residue having a neutral hydrophobic sidechain; X(5) represents an amino acid residue having a neutral polar sidechain; X(6) and X(7) each independently

represent an amino acid residue having neutral aliphatic sidechain; and X(8) represents an amino acid residue having a large hydrophobic sidechain.

40. The preparation of claim 38, wherein: X(1) represents gln, lys, tyr, cyd or thr or
5 an analog thereof; X(2) represents leu, thr, cys, tyr, his, ala or cys or an analog
thereof; X(3) represents met, leu, ile, val, thr or arg or an analog thereof; X(4)
represents leu, lys, his, tyr, trp, arg, val, ile, met, gln or thr or an analog thereof; X(5)
represents gln, ala, ser, cys, asn or tyr or an analog thereof; X(6) represents ala, val or
thr or an analog thereof; X(7) represents leu, ile or val or an analog thereof; X(8)
10 represents leu, tyr, phe, met or gln or an analog thereof; X(9) represents tyr, his or phe
or an analog thereof; X(10) represents lys, tyr, thr or ala or an analog thereof; and
X(11) represents arg or lys or an analog thereof.

41. The preparation of claim 34, wherein the compound comprises a core
15 sequence having the formula: R-X(4)-E-X(5)-X(6)-X(7), wherein:

R represents an arginine residue, or an analog thereof;

E represents a glutamic acid residue, or an analog thereof;

X(4) represents an amino acid residue having a basic sidechain or a neutral
sidechain; and

20 X(5), X(6), and X(7) each independently represent an amino acid residues
having a neutral side chain.

42. The method of claim 41, wherein X(4) represents leu, trp, gln, his, met, or thr,
or an analog thereof; X(5) represents cys, asn, gln or ser, or an analog thereof; X(6)
represents ala, val, or thr, or an analog thereof; and X(7) represents ile, leu, val, or
25 met, or an analog thereof.

43. The preparation of claim 31, wherein the compound is formulated for delivery
into keratinocytes.

44. The preparation of claim 31, wherein the compound is formulated for delivery into cutaneous or mucosal tissue.
- 5 45. The preparation of claim 31, wherein the compound is formulated in liposomes.
46. A method for identifying a compound which inhibits interaction of papillomavirus E1 and E2 proteins comprising.
- 10 (i) generating a reaction mixture comprising the E1 and E2 proteins, or portions thereof which interact, which reaction mixture may be a whole cell, a cell lysate, or a reconstituted protein preparation;
- (ii) contacting the reaction mixture with a test compound, which test compound has a molecular weight of less than 10,000 amu;
- 15 (iii) ascertaining the ability of the test compound to inhibit the interaction of the E1 and E2 proteins.
47. The method of claim 46, wherein the test compound is tested for its ability to inhibit PV replication.
- 20 48. The method of claim 46, wherein the steps are repeated for a library of different test compounds.
49. The method of claim 46, comprising the further steps of formulating a pharmaceutical preparation comprising one or more test compounds which inhibit
25 interaction of the PV E1 and E2 proteins.
50. The method of claim 46, wherein said compounds identified as inhibiting the E1-E2 interaction are further tested in a cell culture based assay.

51. The method of claim 50, wherein said cell culture based assay is a transient replication assay.

5 52. The method of claim 50, wherein said cell culture based assay comprises cultured PV infected cells.

53. A method of treating a mammal for a papillomavirus-induced condition, or preventing said condition, comprising

- 10 (i) identifying a test compound which inhibits interaction of papillomavirus E1 and E2 proteins, which test compound has a molecular weight of less than 10,000 amu;
- (ii) administering, to the mammal, a pharmaceutical preparation comprising a therapeutically effective amount of a test compound which has
- 15 identified as an inhibitor, which compound is formulated in the pharmaceutical preparation for delivery into papillomavirus-infected cells of the animal.

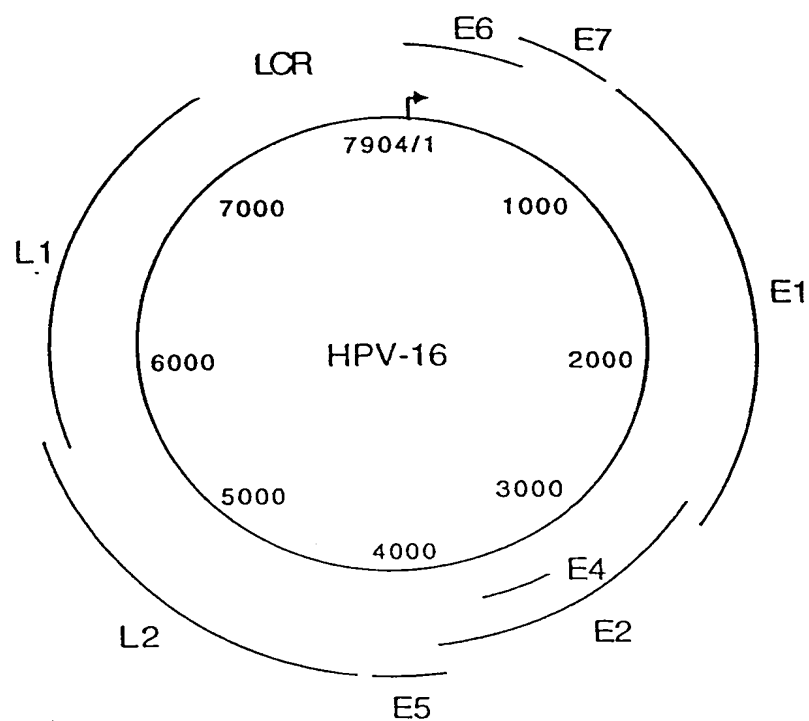


Figure 1

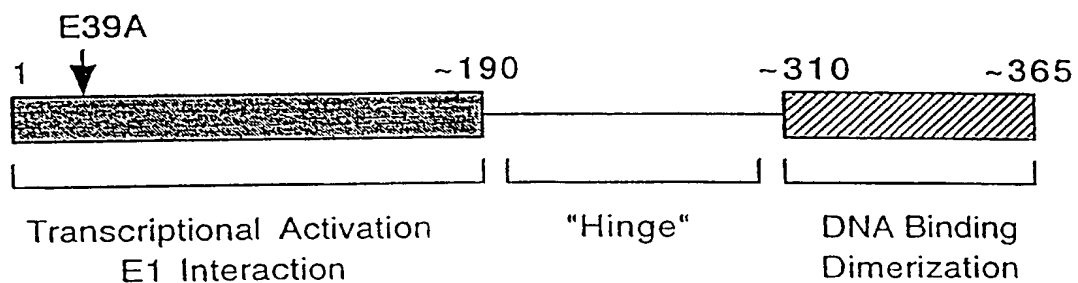


Figure 2

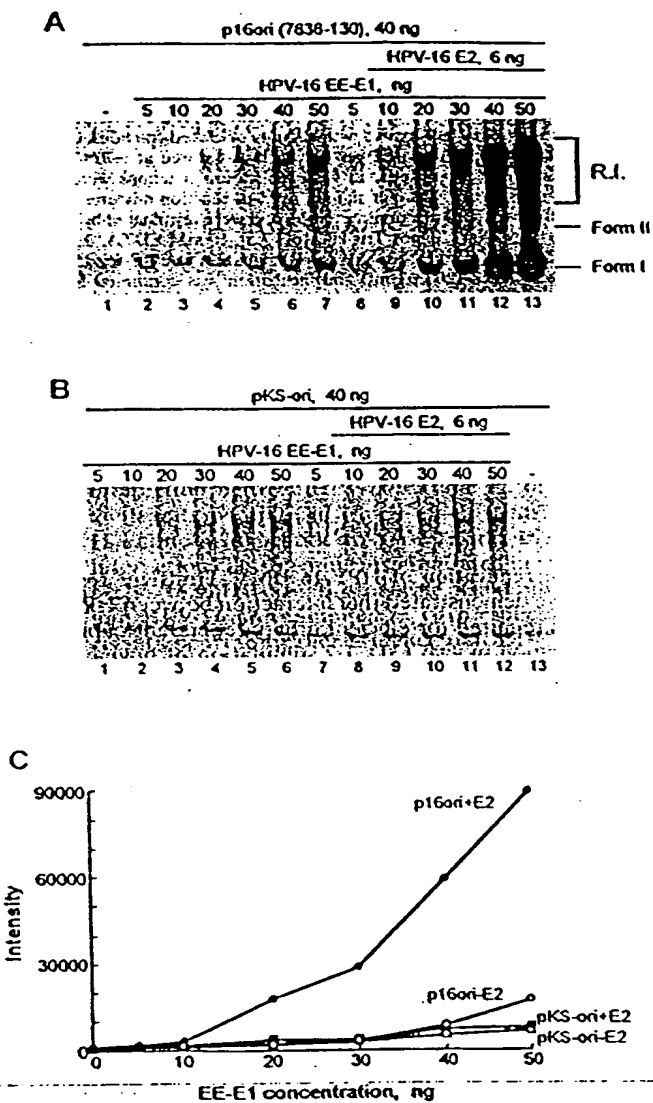


Figure 3

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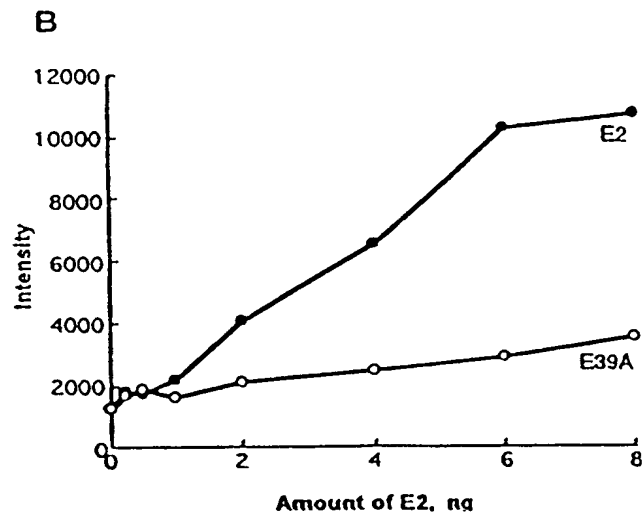
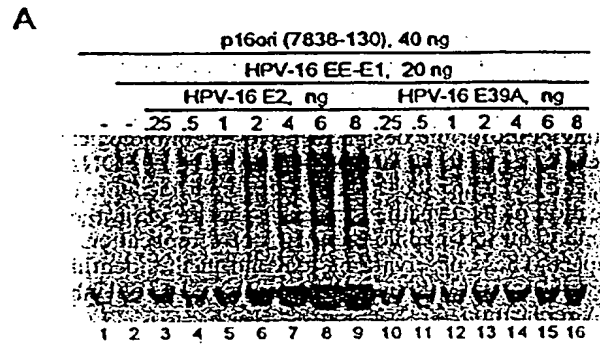


Figure 4

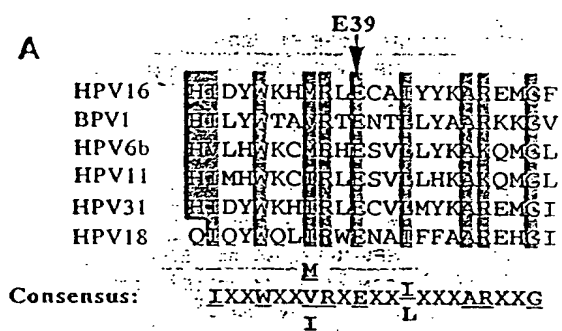


Figure 5A

Figure 5B

W Q L M R L E Q A L L Y K A R
 W Q T L R K E A V L L Y Y A R
 W K C M R H E S V L L Y K A K
 W K C M R H E S V L L Y K A K
 W K Y I R Y E S V I Y Y T A R
 W K C I R L E S V L L H K A K
 W K C L R Y E S V L L H K A R
 W K H M R L E C A I Y Y K A R
 W Q L I R W E N A I F F A A R
 W Q A L R R E A V L L Y Y A R
 W K L V R Y E C A I F Y K A R
 W Y L M R V E S A L Y Y K A R
 W K A V R H E N V V L Y K A R
 W K H I R L E C V L M Y K A R
 W K C L R I E A A L L F K A R
 W K L I R M E C A L L Y T A K
 W K H V R L E N V L L H K A R
 W K L I R L E C A V F Y K A R
 W K C V R M E N A I F Y A A R
 W K Y I R Y E S A I Y Y T A R
 W K C L R M E A V V L Y K A R
 W K C I R Y E C V L L H K A K
 W Q L I R L E N A I L F T A R
 W K L T R M E C V L F Y K A K
 W K A V R Q E N V I Y Y K A R
 W K C I R L E C A L Q Y K A R
 W K C I R Y E C V L L H K A K
 W K L I R M E C A I M Y T A R
 W K A V R H E Y V L Y Y K A R
 W R L R R I E C A L Y Y K A K
 W K Y V R L E N A I F Y A A R
 W C L M R L E S V L L Y K A R

W T A V R T E N T L L Y A A R

W K C V R Y E N V L L H K A R
 W K C V R H E N V L L Y K A R
 type 1
 W K C V R Q E C A V L Y K A R

HPV3
 HPV5
 HPV6a
 HPV6b
 HPV7
 HPV11
 HPV13
 HPV16
 HPV18
 HPV24
 HPV26
 HPV29
 HPV30
 HPV31
 HPV32
 HPV33
 HPV34
 HPV35
 HPV39
 HPV40
 HPV42
 HPV44
 HPV45
 HPV52
 HPV53
 HPV54
 HPV55
 HPV58
 HPV66
 HPV67
 HPV70
 HPV77

BPV1

common chimpanzee PV 1
 Pygmy chimpanzee PV

rhesus monkey PV

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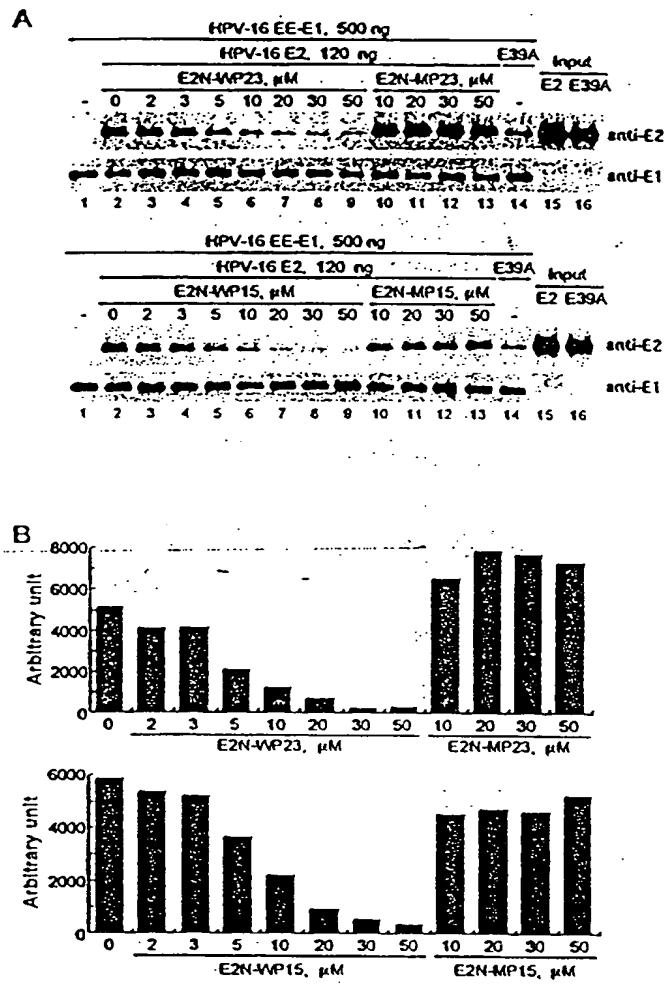


Figure 6

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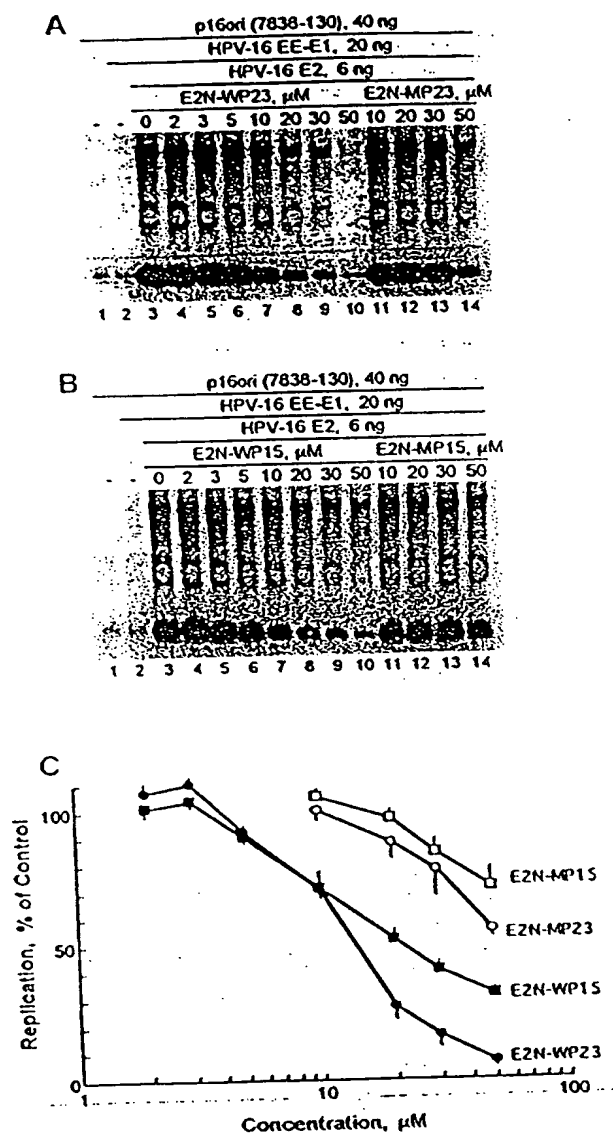


Figure 7

EE-E1 (150ng)											
E2 (30ng)											
Binding Incubation Time						Time of E2N-WP15 Addition					
0	5	10	20	40	60	0	5	10	20	40	
1	2	3	4	5	6	7	8	9	10	11	12

Figure 9

**Papillomavirus
Type**

Amino Acid Sequence

type

33

47

HPV-16	W	K	H	M	R	L	E	C	A	I	Y	Y	K	A	R
HPV-18	W	Q	L	I	R	W	E	N	A	I	F	F	A	A	R
HPV-1A	W	N	L	I	R	Q	E	Q	V	L	F	H	F	A	R
HPV-6b	W	K	C	M	R	H	E	S	V	L	L	Y	K	A	K
HPV-11	W	K	C	I	R	L	E	S	V	L	L	H	K	A	K
HPV-31	W	K	H	I	R	L	E	C	V	L	M	Y	K	A	R
HPV-33	W	K	L	I	R	M	E	C	A	L	L	Y	T	A	K
HPV-35	W	K	L	I	R	L	E	C	A	V	F	Y	K	A	R
HPV-57	W	A	Q	V	R	L	E	N	V	M	L	F	K	A	R
BPV-1	W	T	A	V	R	T	E	N	T	L	L	Y	A	A	R

Figure 12

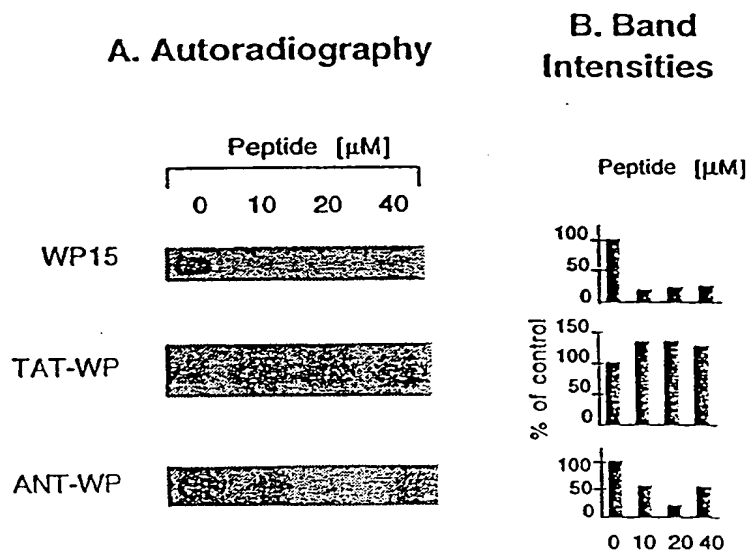


Figure 14

<u>Name</u>	<u>Amino Acid Sequence</u>
WP15	WKHMRLECAIYYKAR
W33A	AKHMRLECAIYYKAR
K34A	WAHMRLECAIYYKAR
H35A	WKAMRLECAIYYKAR
M36A	WKHARLECAIYYKAR
R37A	WKHMALECAIYYKAR
L38A	WKHMRAECAIYYKAR
E39A	WKHMRLECAIYYKAR
C40A	WKHMRLEAAIYYKAR
I42A	WKHMRLECAAYYKAR
Y43A	WKHMRLECAIAYKAR
Y44A	WKHMRLECAIYAKAR
K45A	WKHMRLECAIYYAAR
R47A	WKHMRLECAIYYKAA

Figure 10

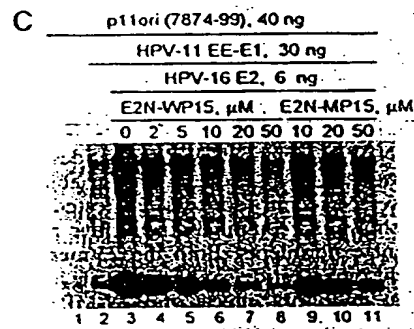
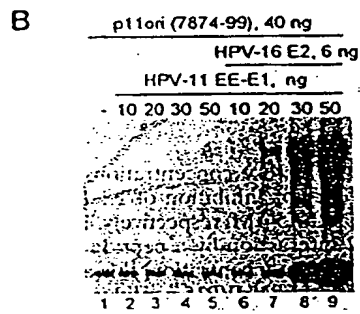
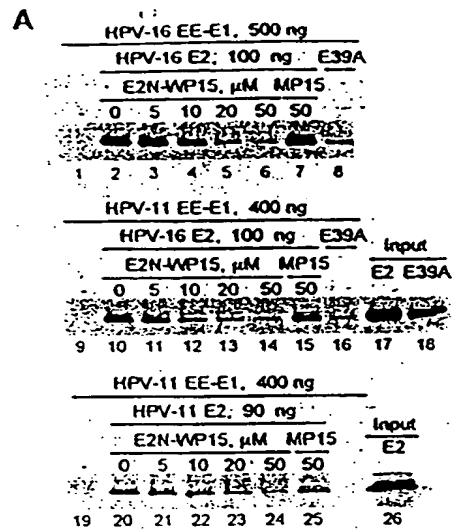


Figure 8

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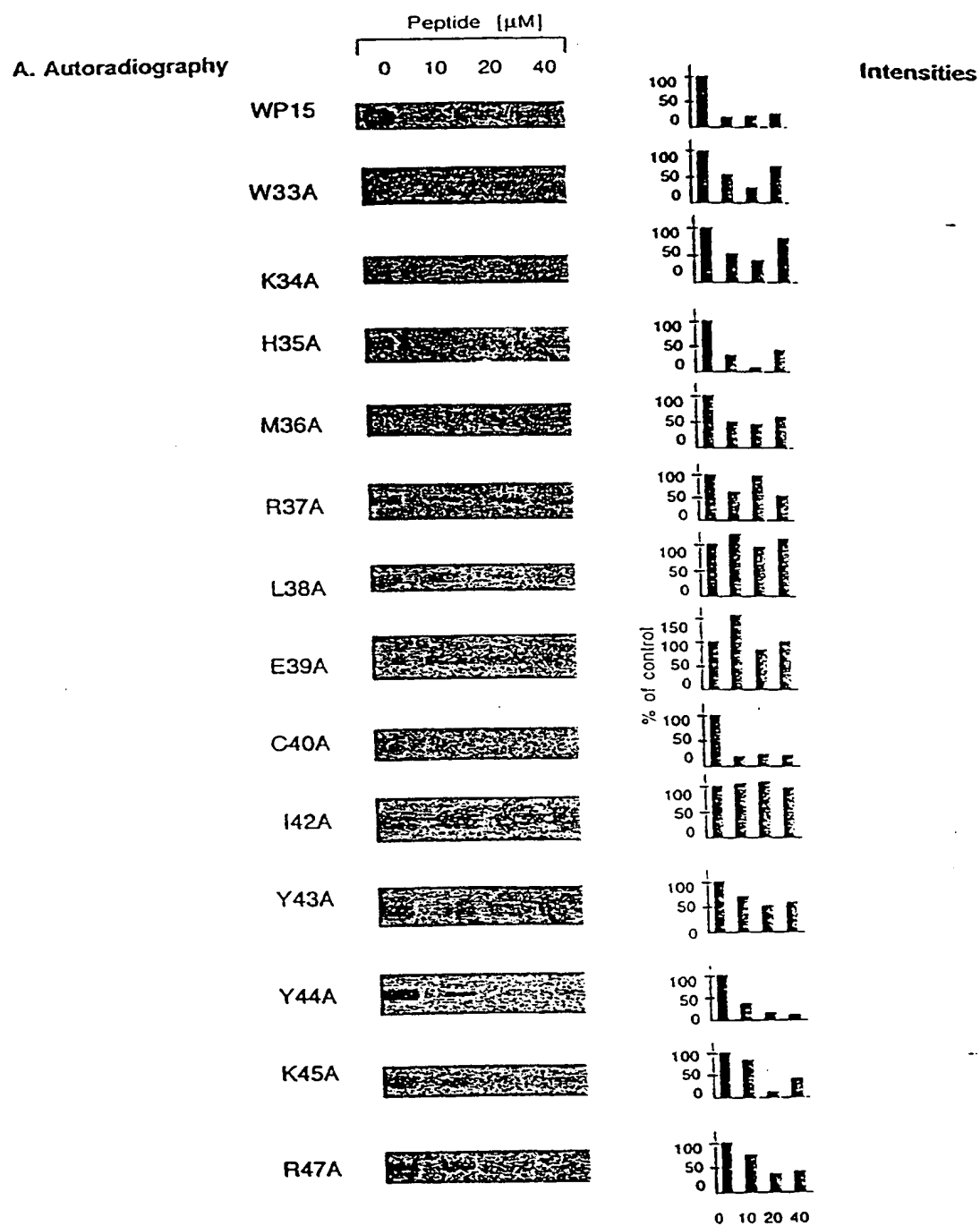


Figure 11

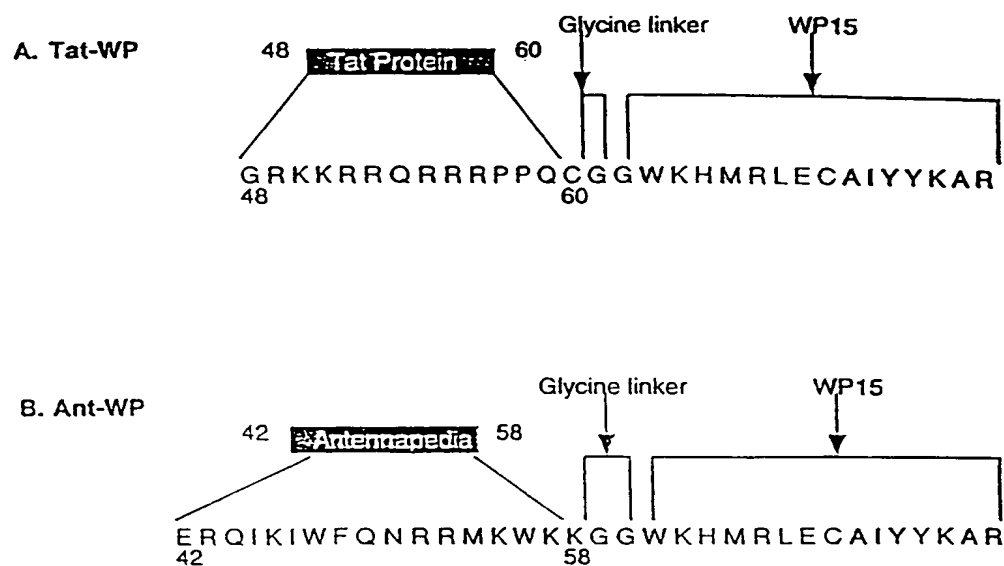


Figure 13

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07K 14/025, G01N 33/50, 33/68, A61K 38/16	A3	(11) International Publication Number: WO 00/01720 (43) International Publication Date: 13 January 2000 (13.01.00)
(21) International Application Number: PCT/US99/15144 (22) International Filing Date: 2 July 1999 (02.07.99) (30) Priority Data: 60/091,661 2 July 1998 (02.07.98) US (71) Applicant: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02138 (US). (72) Inventors: HOWLEY, Peter; 5 Hundreds Circle, Wellesley, MA 02181 (US). BENSON, John; Apartment 3, 118 Franklin Street, Brookline, MA 02445 (US). KASUKAWA, Hiroaki; 16-C Andover Circle, Princeton, NJ 08540 (US). (74) Agents: VINCENT, Matthew, P. et al.; Foley, Hoag & Eliot, LLP, Patent Group, One Post Office Square, Boston, MA 02109 (US).		(81) Designated States: AU, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 17 February 2000 (17.02.00)
(54) Title: COMPOSITIONS AND METHODS FOR TREATING PAPILLOMAVIRUS-INFECTED CELLS (57) Abstract By virtue of the present invention, there is provided methods and compositions for interfering with the proliferation of cells infected and/or transformed by papillomaviruses. The processes and compositions of this invention may be used to treat any mammal, including humans. According to this invention, mammals are treated by the pharmaceutically acceptable administration of an E2 peptidomimetic to reduce the symptoms of the specific papillomavirus-associated disease, or to prevent their recurrence.		

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/15144

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/025 G01N33/50 G01N33/68 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 41018 A (PRESIDENT AND FELLOWS OF HARVARD COLLEGE, USA) 19 December 1996 (1996-12-19) The whole document; see especially claims 15-19 and SEQ ID No.2 ---	1-3, 6-32, 34-53
X	WO 92 11290 A (CETUS CORP ;UNIV CALIFORNIA (US)) 9 July 1992 (1992-07-09) cited in the application The whole document; see especially page 17, lines 10-22 --- -/--	1-8, 15-36, 41-53

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

14 December 1999

Date of mailing of the international search report

28/12/1999

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/15144

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	KASUKAWA H ET AL: "A fifteen-amino-acid peptide inhibits human papillomavirus E1-E2 interaction and human papillomavirus DNA replication in vitro." JOURNAL OF VIROLOGY, (1998 OCT) 72 (10) 8166-73. , XP002125487 the whole document	1-53
A	WO 93 07299 A (CETUS ONCOLOGY CORP ; REAGENTS OF THE UNIVERSITY OF (US)) 15 April 1993 (1993-04-15)	
A	SAKAI E.A.: "Targeted mutagenesis of the HPV type 16 E2 transactivation domain reveals separable transcriptional activation and DNA replication functions" JOURNAL OF VIROLOGY., vol. 70, no. 3, March 1996 (1996-03), pages 1602-1611, XP002125488 ICAN SOCIETY FOR MICROBIOLOGY US cited in the application the whole document	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 15144

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-30 and 53 encompass or are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
claims 1-8,13-36,41-45 (all partially)

see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-8,13-36,41-45(all partially)

The scope of the claims 1-8,13-36,41-45 is unclear and speculative. They lack any structurally defined entity or a very generally defined peptide structure and/or any indication concerning the size of the compound or peptide (Art.6 PCT).

Furthermore the available experimental data relate to only a few peptides. Therefore the claims can also not be considered to represent a permissible generalisation which is fairly based on experimental evidence, that is, they are also not adequately supported and/or disclosed by the description (Artt.5 and 6 PCT).

It is considered that a lack of clarity (and/or conciseness) and a lack of support and disclosure within the meaning of Articles 5 and 6 PCT arise to such an extent as to render a meaningful search of said claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and/or concise), supported and disclosed, namely the compounds of the examples and closely related analogues as defined in claim 6 and their use(Art.17(2)(b) PCT) .

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 99/15144

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9641018 A	19-12-1996	US 5821048 A	13-10-1998
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